1997

Roles of Phospholipase D and Sphingomyelinase in T-Cell Signal Transduction

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ROLES OF PHOSPHOLIPASE D AND SPHINGOMYELINASE IN T-CELL SIGNAL TRANSDUCTION

by

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A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

Molecular and Cellular Biology and Pathobiology Program

1997

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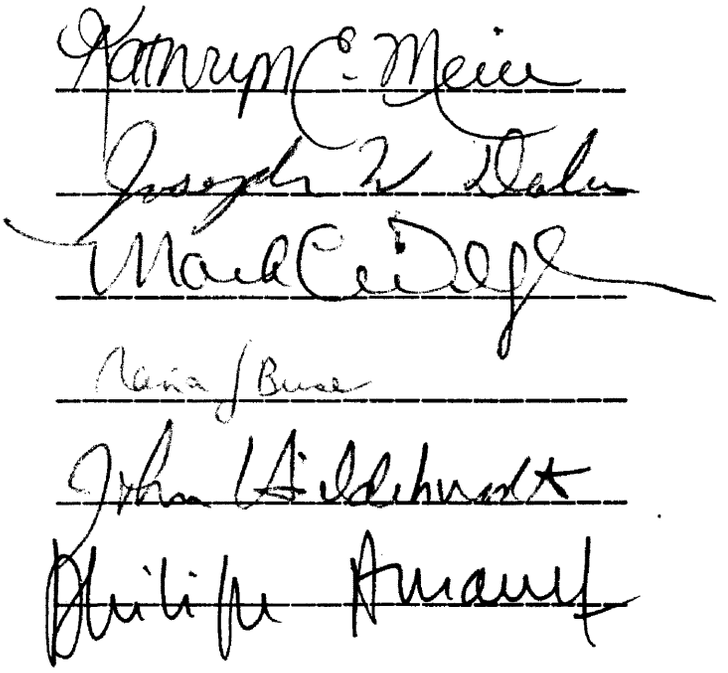

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

My heartfelt appreciation and gratitude to *Dr. Kathryn E. Meier* for her many encouraging words, guidance and support in my educational endeavors;

My committee members, *Dr. Philippe Arnaud, Dr. Maria Buse, Dr. Joseph Dolan, Dr. John Hildebrant, Dr. Mark Willingham, and Dr. Barry Ledford* for their encouraging words of wisdom;

The Office of Diversity for making my educational experience at the Medical University of South Carolina more enjoyable;

Members of the Meier Lab for their friendships, encouragements and support;

My loving parents, *Joseph and Ruthie Bradshaw* for their many prayers, belief in my abilities even when I doubted myself, and for being there when I needed a shoulder to lean on;

My brother, *William* for always trying to keep me in line by giving advice;

A special friend, *Stanley Wilder* for all of his encouragements

and help in difficut times;

My *grandparents, family, and many friends* for their prayers and support;

and of course, God, who gave the strength and perseverance to strive for the best in life.

ABBREVIATIONS

AA	arachidonic acid
AMP	adenosine monophosphate
Ara-C	1-β-D-arabinofuranosylcytosine
ARF	ADP-ribosylation factor
ASK	apoptosis signal-regulating kinase
ATP	adenosine triphosphate
BSA	bovine serum albumin
BPC	2-decanoyl-1-(0-(11-(4,4-difluoro-5,7dimethyl -4-bora-3a,4a-diaza-s- indacene-3-propionyl) amino)undecyl)sn-glycero-3-phospho-choline
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
BSM	N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosyl-phosphocholine
CAPK	ceramide-activated protein kinase
CAPP	ceramide-activated protein phosphatase
CDP	cytosine diphosphate
CMP	cytosine monophosphate
CTP	cytosine triphosphate

DAG	1,2-diacylglycerol
DG	diglyceride
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis[β-aminoethyl ether]-N,N,N'N'-tetraacetic acid
ERK	extracellular signal-regulated kinase
GAP	GTPase-activating protein
GDI	GTP dissociation inhibitor
GDP	guanosine 5'-(2-0-thiol) diphosphate
GST	glutathione S-transferase
GTP	guanosine 5'-3-0-thiol) triphosphate
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piper-azineethanesulfonic acid
HOG1	high osmolarity glycerol 1
IL-1	interleukin-1
IL-2	interleukin-2
IP_3	inositol 1,4,5-triphosphate

JNK	c-Jun N-terminal Kinase
JNKK	c-Jun N-terminal kinase kinase
KDa	kilodalton
LPA	lysophosphatidic acid
MAP-2	microtubule-associated protein-2
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MBP	myelin basic protein
MEK	mitogen-activated protein kinase kinase
MEKK	MAP/ERK kinase kinase
MES	2-[N-morpholino]ethane sulfonic acid
MKK4	mitogen-activated protein kinase kinase 4
MKK6	mitogen-activated protein kinase kinase 6
NPD	Neimann-Pick Disease
PA	phosphatidic acid
PAF	platelet-activating factor
PAK	p^{21}-activated protein kinase
PBt	phosphatidylbutanol
PBS	phosphate-buffered saline

PC	phosphatidylcholine
PC-PLC	phosphatidylcholine-specific phospholipase C
PGE_2	prostaglandin E_2
PI	phosphatidylinositol
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PMSF	phenyl methylsulfonyl fluoride
PP2A	protein phosphatase 2A
PYK	protein tyrosine kinase
Rb	retinoblastoma
RSK	ribosomal S6 kinase
RT-PCR	reverse transcriptase-polymerase chain reaction
SAP	sphingolipid activator protein
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel

	electrophoresis
SEK	stress-activated protein kinase kinase
SEKK	stress-activated protein kinase kinase kinase
SMase	sphingomyelinase
TCR	T-cell receptor
TLC	thin layer chromatography
TNF-α	tumor necrosis factor-alpha
WT	wild type
α	alpha
β	beta
δ	delta
ε	epsilon
η	eta
γ	gamma
λ	lamda
μ	mu
θ	theta
ζ	zeta

LISTS OF FIGURES

ABSTRACT

CYNTHIA DENISE BRADSHAW. Roles of Phospholipase D and Sphingomyelinase in T-Cell Signaling. (Under the direction of KATHRYN E. MEIER, Ph.D).

The overall goal of this study was to investigate the roles of PLD and neutral SMase in signal transduction in T-lymphocytes. In particular, the abilities of phospholipid metabolites to regulate mitogen-activated protein kinases were explored. The tumor-promoting PKC activator, phorbol 12-myristate 13-acetate (PMA), and an antileukemic drug, 1-ß-D arabinofuranosylcytosine hydrochloride (Ara-C), were used as model phospholipase activators.

The effects of phorbol ester and Ara-C on PLD and SMase activities were examined in EL4, a murine thymoma cell line, and Jurkat, a human leukemia cell line. PLD activity was measured in intact cells using a metabolic labelling assay. PLD and SMase activities were measured in membrane extracts utilizing fluorescent phosphatidylcholine (PLD) and sphingomyelin (SMase) as

substrates, respectively. Rapid activation of PLD by PMA was detected in Jurkat cells, but not in EL4 cells. Jurkat, but not EL4 cells, express a 120-kDa protein recognized by an anti-PLD antibody. Ara-C activated neutral SMase within 10 minutes in both Jurkat and EL4. PMA did not activate SMase and Ara-C did not activate PLD.

The relationships between activation of PLD, SMase and members of the mitogen-activated protein kinase family, ERKs (extracellular-regulated kinases) and JNKs (c-jun N-terminal kinases), were examined using *in vitro* phosphorylation assays. PMA induced activation of ERKs in both Jurkat and EL4 cells, while Ara-C or ceramides had no effect. Incubation of EL4 cells with bacterial PLD increased phosphatidic acid levels, but did not activate ERKs. JNK activity was detected within 10 minutes after co-stimulation with PMA and ionomycin in both EL4 and Jurkat cells. Ara-C activated JNKs in these cells only after prolonged incubation (90-120 minutes). These results suggest that activation of PLD is neither necessary nor sufficient for activation of ERKs or JNKs. The delayed effects of Ara-C on JNK activity may be mediated through secondary response pathways.

The mechanism by which PMA activates PLD was examined in

Jurkat cells and in a prostate cancer cell line. No evidence was obtained for PLD translocation, phosphorylation, or binding to protein kinase Cα. These results are consistent with observations that PLD can be activated by protein-protein and/or protein-lipid interactions.

The data obtained in these studies indicate that PLD and neutral SMase are activated via distinct pathways in T-lymphocyte cell lines. While their reaction products do not directly regulate mitogen-activated protein kinases, the activation of these phospholipases by mitogenic and cytotoxic stimuli suggest that further understanding of the regulation of PLD and SMase and their roles in signal transduction may present future avenues for the improved therapy of T-lymphocyte pathologies.

CHAPTER 1

General Introduction

1.1 Phospholipases and Lipid Mediators in Signal Transduction

Lipid mediators play crucial roles in signal transduction. These molecules are considered important in cell proliferation, and in processes involving cell-cell communication including host defense and inflammation (Serhan *et al.,* 1996). The activation of specific phospholipases within individual cell types, mediated by extracellular signals generated during these multicellular processes, is the initiating step in the biosynthesis of several structurally diverse lipid mediators (Billah *et al.,* 1990; Serhan *et al.,* 1996). Phospholipases involved include phospholipase A_2 (PLA_2), phospholipase C (PLC), phospholipase D (PLD), and sphingomyelinase (SMase), each of which is pivotal in the biosynthesis of lipid mediators (Billah *et al.,* 1990; Serhan *et al.,* 1996). Activation of these enzymes is accomplished by a wide range of agonists. The lipid mediators produced include free fatty acids, lysophospholipids, platelet activating factor (PAF), diglyceride (DAG), phosphatidic acid, ceramide, and oxygenated fatty acid metabolites (Serhan *et al.,* 1996). These metabolites have been implicated as essential mediators in a variety of tissue and cell functions. Each major

group of phospholipase (Figure 1-1) contains multiple family members that carry out similar reactions, but are differentially regulated and can act on different substrates. This diversity provides alternative pathways for the highly controlled process of phospholipid degradation and metabolism (Serhan *et al.,* 1996).

PLA_2 hydrolyzes several types of phospholipids to liberate free fatty acids and lysophospholipids, which may either exert direct effects or serve as substrates for the generation of other lipid messengers such as eicosanoids, lysophosphatidic acid, or platelet-activating factor (Balsinde *et al.,* 1996). Of particular interest is the release of free arachidonic acid (AA), which serves as the presursor for numerous bioactive eicosanoid products (Serhan *et al.,* 1996). Mammmalian cells have been shown to contain multiple structurally-diverse PLA_2 enzymes (Dennis, 1994). At least three different cellular PLA_2s have been shown to play a role in the mobilization of AA from phospholipids. These include cytosolic group IV PLA_2s (Lin *et al.,* 1993; Kramer *et al.,* 1993; Qiu *et al.,* 1993), the secretory group II PLA_2s (Murakami et al., 1993; Barbour *et al.,* 1993; Pfeilschifter, 1993), and a cytosolic calcium-independent PLA_2 (Lehman *et al.,* 1993; Ramanadham *et al.,* 1993).

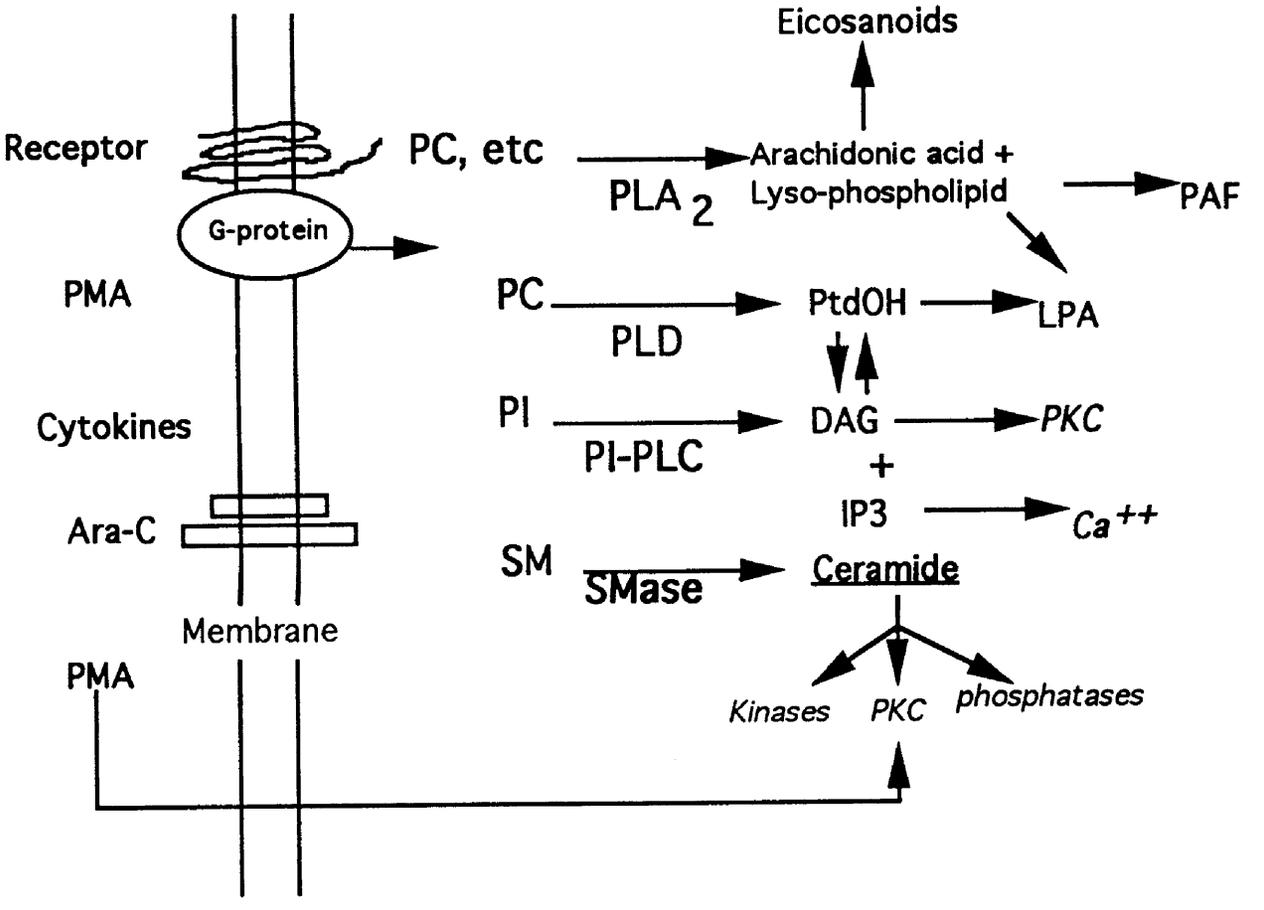

FIGURE 1-1. Overview of Lipid Mediator Function. Each phospholipase is thought to be independently activated to generate specific lipid-derived second messengers. The relative abundance of each pathway's enzymes and precursors are cell-specific. Modified from: Serhan, C.N. Haeggstrom, J.Z., and Leslie, C.C. FASEB J. 10; 1147-1158, 1996.

Cytosolic calcium-dependent PLA_2 is a substrate for protein kinase C (PKC), cyclic-AMP-dependent kinase, and ERK mitogen-activated protein kinase (MAPK) *in vitro* (Wijkander and Sundler, 1992; Lin *et al.*, 1993; Nemenoff *et al.,* 1993; de Carvalho *et al.*, 1996). It is activated when phosphorylated by ERKs (Sa *et al.,* 1995; Clark et al., 1995). Potential regulatory mechanisms for other forms of PLA_2 have not been clearly defined.

PI-specific PLC hydrolyzes inositol phospholipids to generate two second messengers, inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Exton, 1994). IP_3 interacts with a receptor on the endoplasmic reticulum to release calcium from intracellular stores (Bornfeldt *et al.,* 1995). DAG is responsible for activating a large family of PKC isoenzymes that catalyze protein phosphorylation (Nishizuka, 1992; Bruch, 1996). Multiple isoforms of PLC have been identified in a variety of mammalian tissues. The different isoenzymes are differentially regulated by receptor-tyrosine kinases, non-receptor tyrosine kinases, G-proteins, and calcium (Cockcroft *et al.,* 1992; Sternweis and Smroka, 1992).

Phospholipase D (PLD) and sphingomyelinase (SMase), the topics of this thesis project, will be discussed in detail below.

1-2. Protein Kinase C

Protein kinase C (PKC) consists of a family of related isoforms of serine/threonine-specific phospholipid-dependent kinases (Nishizuka, 1992; Blum *et al.*, 1994). PKCs are central elements in cellular signaling. Agonist-induced membrane phospholipid degradation is required for PKC activation (Blum *et al.*, 1994). This step allows information from extracellular signals to be transmitted across the membrane. Activation of PKCs are essential for long term cellular responses such as proliferation and differentiation (Blum *et al.*, 1994). Since PKCs play regulatory functions in signal transduction (Nishizuka, 1986; Brook *et al.*, 1991; Iwashita and Kobayashi, 1992), gene expression (Bernstein *et al.*, 1991; Trejo and Brown, 1991), and cell proliferation and differentiation (Felipo *et al.*, 1990; Minana *et al.*, 1991), these enzymes are considered important in tumorigenesis and are a potential target of new cancer therapies (Blum *et al.*, 1994).

PKCs exist as a family of 11 isoforms that have related structures, but differ with respect to tissue specificity, developmental expression, and subcellular distribution (Newton, 1995). The isoenzymes have been grouped into three functional

classes based on their biochemical and structural properties and cofactor regulation (Newton, 1995; Nishizuka, 1995). The best-described and first-discovered isoforms are the conventional PKCs (cPKCs), α, ßI, ßII, and γ (Liscovitch and Cantley, 1994; Newton, 1995). cPKCs require either phospholipids, calcium, and DAG (or tumor-promoting phorbol esters) for activity (Liscovitch and Cantley, 1994; Newton, 1995). The next well-characterized group is the novel PKCs, which include of δ, ε, η (L), θ, and μ (Liscovitch and Cantley, 1994; Newton, 1995). This group does not require calcium for activity. The least understood izoenzymes are the atypical PKCs (aPKCs) which consist of ζ and λ (I) (Liscovitch and Cantley, 1994; Newton, 1995). This group is not regulated by DAGs or phorbol esters (Liscovitch and Cantley, 1994; Newton, 1995). Each class of PKCs appears to play distinct roles in controlling major cellular functions.

1-3. Phospholipase D

Phospholipase D (PLD), first described in carrot roots and cabbage leaves by Hanahan and Chaikoff (Heller, 1978), catalyzes the degradation of phosphatidylcholine to phosphatidic acid plus choline

in the presence of water. All early studies concerning the PLD reaction were performed in plants. Subsequent studies have shown that PLD is expressed in microorganisms, mammals and yeast. Phosphatidylcholine-preferring PLD can be detected in homogenates and membranes from various mammalian tissues including lung, liver, adipose tissue, endothelium and spermatozoa, with lung and brain being the richest sources (Heller, 1978; Bocckino *et al.*, 1987; Tettenborn and Mueller, 1988; Martin, 1988; Anthes *et al.*, 1991; Geny *et al.*, 1993; Stuchfield and Cockcroft, 1993). In plants and microorganisms, the enzyme is found to exist in both particulate and soluble forms (Heller, 1978). In mammalian cells, PLD activity is found to be associated primarily with particulate fractions (Heller, 1978). However, one group was able to detect PLD activity in the cytosol of various bovine tissues including lung, brain, spleen, heart, kidney, thymus, and liver as well as in rat lung (Wang *et al.*, 1991). The different forms of PLD catalyze the same general reaction (Heller, 1978; Liscovitch, 1989):

Phosphatidyl-R' + R"-OH ⟶ Phosphatidyl-R" + R'-OH

When R" = H, PLD catalyzes a hydrolysis reaction to produce phosphatidic acid. When R"-OH is an alcohol, PLD catalyzes a transphosphatidylation reaction to produce phosphatidylalcohol. A variety of alcohols can serve as substrates (Ella *et al.,* 1997). PLD is unique from other phospholipases with respect to its broad substrate specificity, as well as in its ability to catalyze the "transphosphatidylation" reaction (Heller, 1978; Kobayashi and Kanfer, 1987). Both hydrolysis and transphosphatidylation occur via a common phosphatidyl-PLD complex formed by the nucleophilic attack by PLD at the phosphorus of the substrate (Heller, 1978; Kobayashi and Kanfer, 1987). The alcohol binding site associated with the transphosphatidylation reaction appears to be largely conserved between plant, yeast, and mammalian PLDs (Morris *et al.,* 1996).

1-4. Regulation of Phospholipase D

The activation of PLD has been implicated as a crucial step in a broad spectrum of physiological processes and diseases, including metabolic regulation, inflammation, secretion, mitogenesis, oncogenesis, neural and cardiac stimulation, diabetes, and

senescence (Exton, 1994). Since PLD has been identified as a potentially important component of these processes, its modes of regulation are under intense investigation.

In many cell types, PLD is activated in response to a diverse array of growth factors and mitogenic agonists (Morris *et al.,* 1996). PLD has been shown to be activated by the same agonists that activate phosphatidylinositol- and phosphatidylcholine-utilizing PLCs (PIP_2-PLC; PC-PLC) (Thompson, 1991). Reports have suggested that activation of PLD can occur through multiple mechanisms involving PKC, calcium ions, tyrosine kinases and/or GTP-binding proteins such as Ras (Cockcroft, 1992; Exton, 1994), ARFs (Brown, 1993; Massenburg *et al.,* 1994; Cockcroft *et al.,* 1994), Rho A and B, Rac and Cdc42 (Bowman *et al.,* 1993; Exton, 1994; Siddiqi *et al.,* 1995).

There appear to be multiple isoforms of PLD. Studies in numerous mammalian tissues and cell lines have identified PLD activities that differ in their subcellular localization, pH optima, dependence on divalent cations, and susceptibility to inhibition by fatty acids (e.g. oleate) and detergents (Morris *et al.,* 1996). These results suggest the existence of PLD isozymes that differ with

respect to activation mechanism, substrate specificity, and co-factor requirements (Exton, 1994). Studies in Leydig cells have shown that PLD activated by phorbol 12-myristate 13-acetate (PMA) preferentially hydrolyzes ester-linked phosphatidylcholine, whereas PLD activated by a G-protein-coupled receptor does not discriminate between ether- and ester-linked phosphatidylcholine (Lauritzen and Hansen, 1995). In contrast, Huang and co-workers (1992) using MDCK cells, showed that PMA-activated PLD selectively hydrolyzes ether-linked phosphatidylcholine, while ARF- or Rho-regulated PLD preferentially hydrolyses ester-linked phosphatidylcholine. Even though most forms of PLD prefer phosphatidylcholine, it has been shown that the cytosolic form of PLD not only hydrolyzes phosphatidylcholine, but also phosphatidylethanolamine and phosphatidylinositol (Wang *et al.,* 1991). Phosphatidylserine and phosphatidylethanolamine are both utilized by a recently-described yeast PLD that is calcium-dependent and does not perform a phosphatidylation reaction (Mayr *et al.,* 1996).

The involvement of PKC in the activation of PLD has been demonstrated by several approaches. Treatment of cells with phorbol 12-myristate 13-acetate (PMA), an activator of PKCs, can

activate PLD but not PLC. More recently, a role for specific PKC isozymes in the regulation of PLD was shown by studies in which PKCα or -ß were overexpressed (Ohguchi *et al.,* 1996). Overexpression of PKCßI enhances PMA-induced PLD activity in rat fibroblasts (Pai *et al.,* 1991). Overexpression of PKCα in Swiss-3T3 fibroblasts (Eldar *et al.,* 1993) elevates basal and agonist-stimulated PLD activity in intact cells as well as *in vitro.* In membranes isolated from CCL39 fibroblasts (Conricode *et al.,* 1994), only PKCα and -ß are capable of activating PLD. These results suggest that activation of PLD may be selectively mediated by particular PKC isoforms.

The exact mechanism by which PKC activates PLD is not fully understood. It is possible that PKC directly interacts with PLD in membranes, or that PKC interacts with other membrane-associated proteins that in turn activate PLD (Ohguchi *et al,* 1996). Conricode an co-workers (1994) have shown that PMA activation of PLD involves PKC but does not require ATP-dependent phosphorylation in Chinese hamster lung fibroblasts. According to Singer and co-workers (1996), PKC may regulate signaling events through direct molecular interaction with downstream effectors as well as through

its well-characterized catalytic modification of proteins by phosphorylation.

In cell-free preparations from several tissues, PLD activity is enhanced by the addition of calcium. Calcium is also required for GTPγS-stimulated PLD activity in granulocyte homogenates (Anthes *et al.*, 1991; Xie and Dubyak, 1991) and for ATP-induced phosphocholine formation in hepatocyte membranes (Irving and Exton, 1987). By contrast, in several preparations, including hepatocyte membranes, homogenates from endothelial cells (Bocckino *et al.*, 1987; Martin, 1988), spermatozoal extracts (Domino *et al.*, 1989), and some T-lymphocytes, PLD activities do not require calcium. This suggests that calcium requirement for PLD may vary among cell types, that some forms of PLD may require calcium, and/or that calcium may be indirectly effecting PLD through some unknown mechanism. Mayr and co-workers (1996) have identified a strictly calcium-dependent PLD in *Saccharomyces cerevisiae*. This 60kDa PLD preferentially hydrolyzes phosphatidylserine and phosphatidylethanolamine. Unlike previously-characterized plant, yeast, and mammalian PLDs, this calcium-dependent PLD is not affected by PIP_2 and does not catalyze a transphosphatidylation

reaction in the presence of alcohol (Mayr *et al.,* 1996). It appears that this calcium-dependent PLD is a member of a novel family of PLDs. Since mammalian PLDs are usually assayed through use of the transphosphatidylation reaction, it is not yet clear whether a similar calcium-dependent enzyme is expressed in mammalian cells.

A strong correlation between tyrosine phosphorylation and PLD activation was established in studies performed by Bourgoin and Grinstein (1992), in which treatment of permeabilized HL-60 cells with vanadyl hydroperoxide induced the accumulation of tyrosine-phosphorylated proteins along with an increase in PLD activation. Uings and co-workers (1992) showed that three tyrosine kinase inhibitors blocked the activation of PLD induced by several agonists in neutrophils, whereas pervanadate increased tyrosine phosphorylation and PLD activity. These results suggest that PLD can be activated by an increase in tyrosine phosphorylation, thereby providing a role for tyrosine kinases in the regulation of PLD.

Monomeric G proteins have been implicated as regulators of PLD in many mammalian cell types. Studies by Carnero and co-workers (1994) have shown that, in NIH3T3 fibroblasts, mitogenic stimulation induced by Ras oncogenes is functionally linked to

activation of PC-PLD. Several other groups have identified ARF proteins as effective activators of PLD in HL-60 cells (Brown *et al.,* 1993; Cockcroft *et al.,* 1994b). ARF is a 21 kDa guanine nucleotide-binding protein that is responsible for enhancing the cholera toxin catalyzed ADP-ribosylation of the $G_s\alpha$ subunit that stimulates adenylate cyclase (Ding *et al,* 1996). ARF has also been identified as an essential component of pathways for protein trafficking in cells.

The small GTP-binding proteins of the Rho family, Rho A and -B, Rac, and Cdc42, are implicated in signal transduction pathways that regulate both the actin cytoskeleton and mitogen-activated protein kinase (MAPK) cascade. The involvement of these proteins in the activation of PLD was demonstrated by Exton and colleagues, who showed that treatment of plasma membranes from rat liver with Rho-GDI reduced guanine nucleotide-stimulated PLD activity (Siddiqui *et al.,* 1995). This activity could however be restored with the addition of purified RhoA protein. Subsequent studies revealed that a partially purified PLD preparation from porcine brain, as well as PLD activities in cytosol and membranes of HL-60 cells, could be activated by ARFs and by Rho A and -B, Rac and Cdc42 (Morris *et al.,* 1996). Rho A has been shown to act synergistically with PKC to

stimulate activation of membrane-associated PLD activty in HL-60 cells (Ohguchi *et al.,* 1995; Ohguchi *et al.,* 1996). Rho A and ARF can also exert a synergistic effect on PLD activation in several cell types, including HL-60 cells (Siddiqui *et al.,* 1995; Morris *et al.,* 1996). Rho-specific GDP dissociation inhibitor can suppress synergistic PLD activation by GTPγs and protein kinase C in HL-60 membranes (Malcolm *et al.,* 1994). These synergisms suggest that Rho A may be cooperatively regulating ARF- and protein kinase C-induced PLD activity in HL-60 cells (Siddiqui *et al.,* 1995). Rac1 has been shown to play a major role in the activation of PLD by epidermal growth factor in Rat1 fibroblasts (Hess *et al.,* 1997). Additional studies have shown that ARF- and Rho-responsive PLD activities are specifically stimulated when phosphatidylinositol 4,5-bisphosphate is included in the substrate-containing phospholipid vesicles (Morris *et al.,* 1996). These results further suggest a role for this phospholipid as a cofactor or mediator of PLD activation.

PLD can be negatively regulated by cellular cytosolic factors. According to Lukowski and co-workers (1996), a major increase in the total PLD activity was observed during an initial step of

purification of cytosolic PLD activators, suggesting the removal of an inhibitor. This group was later able to show that fodrin, a high molecular mass, cytoskeletal protein, was responsible for inhibiting PLD activity. A specific protein from bovine brain cytosol has been identified that potently inhibits PIP_2-dependent PLD activity (Kim *et al.,* 1996). This 30-kDa inhibitor specifically regulates PLD activity, but does not bind substrate vesicles containing PIP_2. In addition, the suppression of PLD activity by the inhibitory protein can be reversed by the addition of ARF. These results provide further evidence for the involvement of cytosolic factors in negatively regulating PLD activity. The role of such factors in regulating PLD activity in intact cells is not clear. PLD activity can also be negatively regulated in intact cells via the heterotrimeric G-protein, Gi (Ella *et al.,* 1997).

Despite the wealth of information concerning the biochemical properties of PLD, relatively little is known about its structural and molecular properties. An attempt to answer these issues led to the identification and characterization of a PC-specific PLD activity in plants (Wang *et al.,* 1994). The cloning of this gene assisted in identification of the gene encoding a phosphatidylcholine-

hydrolyzing PLD1 in the yeast, *Saccharomyces cerevesiae* (Ella *et al.*, 1996). This gene is localized to the site of the SPO14 sporulation mutation (Waksman *et al.*, 1996), and encodes a PLD with transphosphatidylating catalytic activity (Ella *et al.*, 1995; Ella *et al.*, 1996). Additional studies, using yeast PLD1 as a tool, led to the cloning and characterization of human PLD1 (Hammond *et al.*, 1995). hPLD1 is membrane-associated and is activated by phosphatidylinositol 4,5-bisphosphate and by ARF (Hammond *et al.*, 1995). A splice variant of this gene was recently characterized (Hammond *et al.*, 1997). The characterization of each of the PLDs encoded by each of these genes will be essential for revealing structural features of PLDs and their mechanisms of regulation.

1-5. Mechanism of Action of Phosphatidic Acid

Phosphatidic acid (PA), product of the reaction catalyzed by PLD, may act as an intracellular second messenger in PLD-mediated signaling processes. The role of PA has been difficult to establish because this substrate is taken up poorly by cells, and can be metabolized on the outer surface of the cell. Nonetheless, PA is mitogenic for some mammalian cell types. In epithelial cells, PA

has been shown to increase release of calcium from intracellular stores and to stimulate synthesis of DNA (Moolenaar *et al.,* 1986). In 3T3 fibroblasts, PA is highly mitogenic. This effect can be seen following down-regulation of PKC, but is blocked by antibodies to ras proteins (Yu *et al.,* 1988). Additional studies in Balb/c 3T3 cells, using monoclonal antibodies to PA to quantify levels of PA, have also suggested that PA accumulation is important in mitogenesis (Fukami and Takenawa, 1992). All of these studies are complicated by potential metabolism of PA to active metabolites such as LPA.

The molecular targets of PA remain poorly defined. PA can stimulate protein phosphorylation when added to cell extracts (Bocckino *et al.,* 1991; Meier, 1992). In human platelets, a PA-activated protein kinase has been characterized (Khan *et al.,* 1994). PA has been shown to inhibit the GAP-mediated stimulation of the GTPase activity of Ras *in vitro*, as well as to stimulate activation of ARF-GAP proteins (Tsai *et al.,* 1989). PA has also been shown to activate Ras-related p21 Rac-GAP activity (Ahmed *et al.,* 1993). PA can bind to Raf-1 and enhance its activity (Ghosh *et al.,* 1996). However, it has been difficult to address the significance of these

effects in intact cells.

Lysophosphatidic acid (LPA), a product of PA metabolism and a contaminant of PA preparations, can act as an extracellular signal. LPA has been shown to be mitogenic in some cells (Moolenaar *et al.,* 1986), and to activate PLD (Van der Bend *et al.,* 1992). There is increasing evidence that many cells possess a surface receptor for LPA (Thomson *et al.,* 1994). The function of this receptor is often blocked by pertussis toxin, an inhibitor of G_i-mediated signalling (Nagami *et al.,* 1995). Thus, many of the actions of LPA, such as PI hydrolysis, calcium elevation, AA release, inhibition of adenylate cyclase, chemoattraction, and PLD activation, can be attributed to receptor-activated G-protein-mediated mechanisms. LPA can also induce activation of ERK mitogen-activated protein kinases via Ras and Raf (Howe and Marshall, 1993; Hordijk *et al.,* 1994). This action can be mediated by activation of c-Src (Luttrell *et al.,* 1996). LPA is a strong activator of Rho-dependent signal transduction pathways (Ahmed *et al.,* 1993). While LPA can be released by platelets (Ohata *et al.,* 1996) and is a major growth factor in serum (Krebs *et al.,* 1993), its potential roles as an autocrine or paracrine mediator have not been adequately explored.

1-6. Roles of Sphingolipids in Signal Transduction

Based on the examples for glycerol-phospholipids, the roles of sphingolipids in signal transduction have been examined. Sphingolipids are complex lipids that serve a structural role in membranes. Sphingolipids consist of a long chain amino dialcohol base (sphingoid), an amide-linked fatty acyl group, and a polar or glycosidic head group (Hannun and Bell, 1993). The simplest sphingolipid, ceramide, serves as a precursor for more complex sphingolipids (Hannun and Bell, 1993). These include sphingomyelin, a major constituent of most membranes in eukaryotic cells, as well as neutral glycolipids and more complex acidic glycosphingolipids (Hannun and Bell, 1993).

Sphingolipids have roles in the response to cell contact, as receptor components, as anchors for proteins, and as markers of tumor progression and cell differentiation (Hannun and Bell, 1993). Sphingolipids also play a significant role in the regulation of cell viability (Hannun, 1996). In both *Saccharomyces cerevisiae* and mammalian cells, mutations in the first enzyme in *de novo* sphingolipid biosynthesis (serine-palmitoyl transferase) result in abolition of sphingolipid formation and loss of viability that is

reconstituted by replacement with sphingolipids (Hannun, 1996).

A potential role for sphingolipids as signaling molecules became evident with the demonstration that sphingosine, an endogenous sphingolipid metabolite, reversibly inhibits PKC (Hannun *et al.,* 1986). This observation prompted further investigations that led to the discovery of a sphingomyelin cycle of cell regulation and the potential role of ceramide as a second messenger/intracellular mediator (Hannun and Bell, 1989).

1-7. Sphingomyelin Cycle

The sphingomyelin cycle (Figure 1-2) was first characterized by Okazaki and co-workers in human HL-60 leukemic cells (Okazaki *et al.,* 1989). When these cultured cells were labeled with choline and then stimulated with 1-α,25-dihydroxyvitamin D_3, the levels of sphingomyelin changed substantially, indicating hydrolysis of cellular sphingomyelin to generate choline phosphate and ceramide (Okazaki *et al.,* 1989). Other investigations were subsequently performed to examine the components of the sphingomyelin cycle, the hormone-activated enzymes regulating sphingomyelin hydrolysis, the role of sphingomyelin hydrolysis and the generated

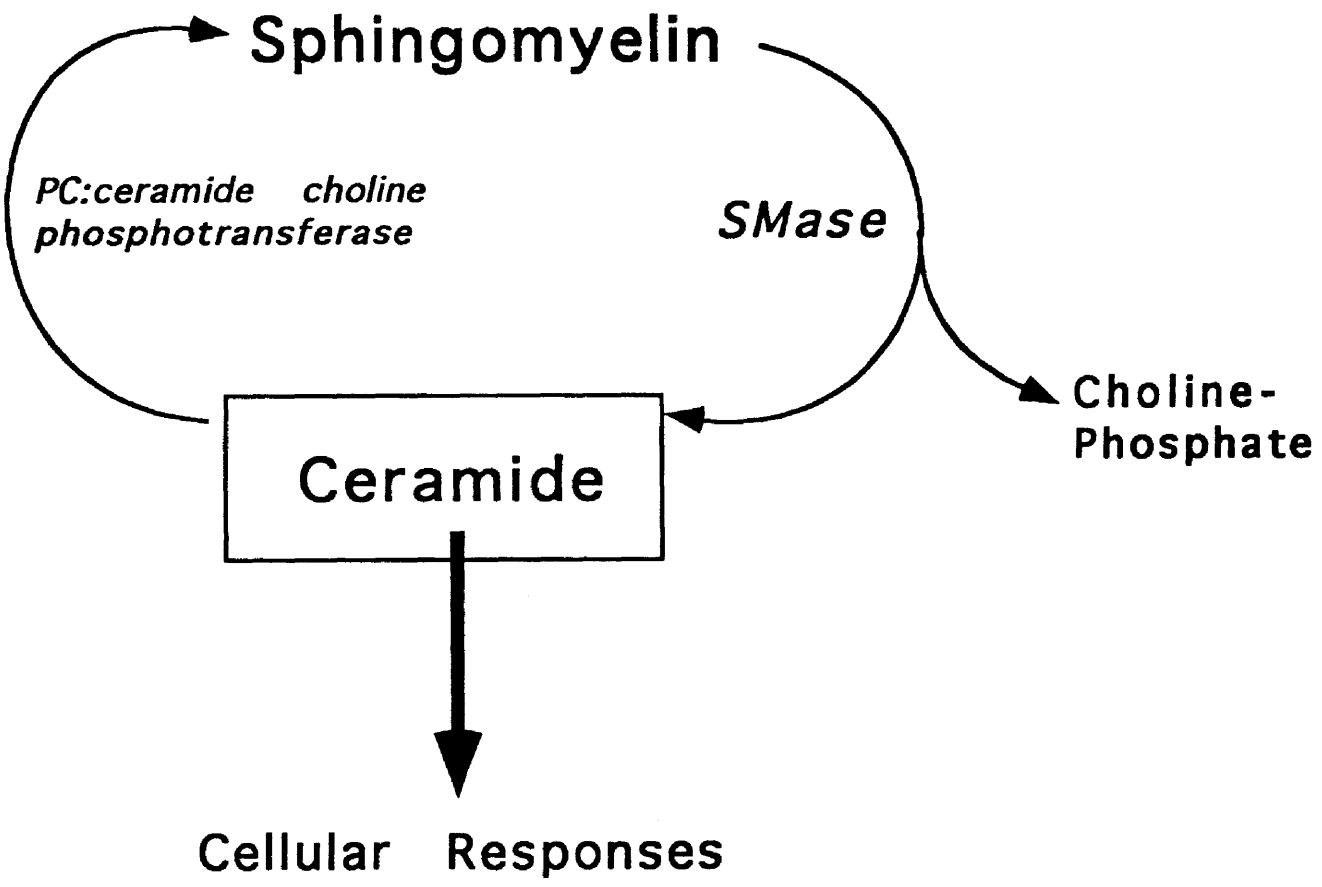

FIGURE 1-2. The sphingomyelin cycle.

Sphingomyelin is cleaved by sphingomyelinase to yield ceramide and choline phosphate. Sphingomyelin is regenerated from ceramide via the transfer of choline phosphate from phosphatidylcholine by action of phosphatidylcholine:ceramide choline phosphotransferase. Adapted from: Saba J. D., Obeid, L.M., and Hannun, Y.A. Philos. Trans. R. Soc. London B. Biol. Science. 351; 233-240, 1996.

products in biological responses, and the extracellular inducers of sphingomyelin hydrolysis (Hannun and Bell, 1993). Evidence obtained thus far suggests that the sphingomyelin cycle is involved in the regulation of cell growth, differentiation, programmed cell death, and cell attachment (Hannun, 1994).

The major components of the sphingomyelin cycle are: a) sphingomyelin, the substrate; b) sphingomyelinase, the enzyme that catalyzes the hydrolysis of sphingomyelin; c) ceramide and choline-phosphate, products resulting from the hydrolysis of sphingomyelin; and d) the enzymatic pathway for the resynthesis of sphingomyelin (Kolesnick and Golde, 1994) (Figure 1-2). The enzymatic pathway for the resynthesis of sphingomyelin from ceramide occurs via the transfer of choline phosphate from phosphatidylcholine by action of phosphatidylcholine:ceramide choline phosphotransferase.

Sphingomyelin is a naturally-occurring membrane lipid that is preferentially localized in the outer leaflet of the plasma membrane of mammalian cells (Kolesnick, 1991). Sphingomyelin is composed of a long chain sphingoid-base backbone (predominantly sphingosine), a fatty acid, and a phosphocholine headgroup (Kolesnick, 1991; Saklatvala, 1995). Sphingomyelin is hydrolyzed

via a PLC-like reaction catalyzed by the enzyme sphingomyelinase (SMase) (sphingomyelin phosphodiesterase) (Zhang and Kolesnick, 1995) into phosphocholine and ceramide. The phosphocholine head group is released into the extracellular environment while ceramide is thought to diffuse within membranes, acting as an intracellular or membrane-localized messenger (Testi, 1996).

Hydrolysis of sphingomyelin to ceramide has been described in a variety of cell lines in response to various stimuli, including 1-α,25-dihydroxyvitamin D_3 (Okazaki *et al.,* 1989; Okazaki *et al.,* 1990), diacylglycerols (Testi, 1996), ionizing radiation (Haimovitz-Friedman *et al.,* 1994), progesterone (Strum *et al.,* 1995), and chemotherapeutic agents (Strum *et al.,* 1994). Accumulation of ceramide derived from sphingomyelin hydrolysis can also result from binding of ligands to cell-surface receptors, including tumor necrosis factor receptor (Dressler *et al.,* 1992; Yanaga and Watson, 1992; Dbaibo *et al.,* 1993; Kim *et al.,* 1994), interleukin-1β receptor (Ballou *et al.,* 1992; Mathias *et al.,* 1993), nerve growth factor receptor (Dobrowsky *et al.,* 1994), FAS/APO-1 antigens (Cifone *et al.,* 1993), and CD28 (Testi, 1996). The mechanisms responsible for coupling these receptors to SMase activation have not been

identified.

1-8. Sphingomyelinases

Sphingomyelinases (SMases), which are responsible for intracellular degradation of sphingomyelin to ceramide, are considered essential components of the sphingomyelin cycle. Several forms of SMase have been identified in variety of mammalian tissues and organs. Acid and neutral SMases were the first two enzymes identified. They are suggested to participate in signal-specific biological processes (Spence, 1994; Chatterjee, 1994). The other forms of SMase include a cytosolic SMase (Okazaki *et al.,* 1994), a zinc-stimulated SMase (Spence *et al.,* 1989; Schissel *et al.,* 1996), and an alkaline SMase (Duan *et al.,* 1995; Nyberg *et al.,* 1996).

Acid SMase is a lysosomal hydrolyase that is ubiquitously distributed in all tissues. Acid SMase acts optimally at pH of 4.5-5.5 *in vitro* (Spence, 1994). This enzyme does not require a cofactor for maximal activity and is not affected by EDTA or Triton X-100 (Kolesnick *et al.,* 1991). Acid SMase activity is, however, inhibited by the thiol reagent dithiothreitol (DTT) and by some lipids and

detergents, such as fatty acids, sphingosine, and sodium dodecyl sulfate (SDS) (Maruyama and Arima, 1989). Kronke and co-workers (Wiegmann *et al.,* 1992; Schutze *et al.,* 1994) have suggested that acid SMase may be activated by some agonists. In this case, acid SMase is activated by DAG generated by a ligand-responsive phosphatitdylcholine-specific PLC (Wiegmann *et al.,* 1992; Schutze *et al.,* 1994). Mutation of acid SMase results in the two major subtypes of human Neimann-Pick Disease (NPD), an inherited disorder characterized by accumulation of sphingomyelin (Schuchman *et al.,* 1991). Type A NPD is a rapidly progressing neurodegenerative disorder (Schuchman *et al.,* 1991). Type B NPD causes manifestations limited to the reticuloendothelial system (Schuchman *et al.,* 1991). Three human acid SMase cDNAs have been isolated and sequenced from fibroblast, placental, testis, hepatoma, and retinal cDNA libraries (Schuchman *et al.,* 1991). Transient expression studies showed that type 1 transcript, the major species, encodes a catalytically active enzyme. Sphingolipid activator proteins (SAPs), or saposins, play a role in the hydrolysis of sphingomyelin via acid SMase (Kolesnick, 1991). Saposins (A, B, C, and D), are a group of homologous glycoproteins derived from a

single precursor, prosaposin, that enhance lysosomal hydrolase action (Kolesnick, 1991; Vaccaro *et al.,* 1995). The action of these proteins was originally described as replacing detergent requirements *in vitro* (Kolesnick, 1991). Saposins A, B, and D have SMase-enhancing capabilities, while saposin C, also known as SAP-2, enhances hydrolysis of sphingomyelin, glucosylceramide, and galactosylceramide by β-glucosylceramidase (Qi *et al.,* 1996).

Neutral SMase is a membrane-bound enzyme that is thought to be co-localized with its substrate on the outer leaflet of the plasma membrane (Ghosh and Chatterjee,1987; Chatterjee, 1993). This enzyme requires divalent cations such as magnesium for activity (Ghosh and Chatterjee,1987; Kolesnick, 1991; Chatterjee, 1993). Neutral SMase has an optimal pH of 7.0-7.5. This enzyme can be activated in response to a number of extracellular stress-related stimuli. High levels of activity can be detected in brain and neural tissues (Chatterjee, 1993). Neutral SMase is believed to be most important in generating lipid messengers to regulate cellular functions including proliferation, differentiation, and apoptosis (Chatterjee, 1993; Hannun, 1994). A neutral, Mg^{2+}-dependent SMase has also been identified in *S. cerevisiae* (Ella *et al.,* 1997).

Okazaki and co-workers (1994) described a cytosolic SMase activated in HL-60 cells upon stimulation with 1-α,25-dihydrovitamin D_3. This enzyme has optimal activity at neutral pH and does not require magnesium for activity. Cytosolic SMase is inhibited by copper, ferric iron, and zinc. Activity of this enzyme is modestly enhanced by phosphatidic acid, phosphatidylserine, or phosphatidylinositol, but not by other major phospholipids (Okazaki *et al.,* 1994). Cytosolic, magnesium-independent SMase is reportly activated during cell differentiation (Okazaki *et al.,* 1994).

Zinc-stimulated SMase, originally described by Spence and co-workers (1989), was detected in fetal bovine serum and, in lower levels, in newborn human serum. Recent studies, have shown that zinc-SMase activity is secreted by human and murine macrophages, human skin fibroblasts, microglial cells, and several other cells in culture (Schissel *et al.,* 1996). Zinc-SMase arises from the acid SMase gene independently of alternative splicing, suggesting a post-translational modification (Schissel *et al.,* 1996). This enzyme has optimal activity at a pH of 5.5 (Spence *et al.,* 1989; Schissel *et al.,* 1996). Zinc-stimulated SMase is inhibited by AMP and EDTA (Schissel *et al.,* 1996). The biological function of zinc-stimulated

SMase is unknown. However, it is hypothesized that this enzyme may play roles in physiological and pathophysiological processes involving extracellular sphingomyelin hydrolysis (Schissel *et al.*, 1996).

Alkaline SMase is thought be a potential player in digestion of dietary sphingomyelin and in gallbladder diseases (Duan *et al.*, 1995; Nyberg *et al.*, 1996). Alkaline SMase has an optimal pH of 9.0 (Duan *et al.*, 1995; Nyberg *et al.*, 1996). This enzyme is enriched in brush-border preparations of rat intestinal mucosa and is present in human duodenal content and meconium (Duan *et al.*, 1995). It is also present in both human hepatic bile and gallbladder bile (Nyberg *et al.*, 1996). The activity of alkaline SMase is dependent on bile salts, but is not increased by Triton X-100. Calcium or magnesium does not change the activity of this enzyme in the presence of bile salts (Nyberg *et al.*, 1996).

1-9. Ceramide: An Intracellular Second Messenger

Ceramide is a central molecule in sphingolipid structure and metabolism (Hannun, 1994). The activation of cellular sphingomyelinase generates ceramide along with phosphocholine.

These products are then recycled back to sphingomyelin, defining the sphingomyelin cycle (Hannun, 1994).

Biological studies utilizing ceramide analogs have indicated that ceramide possesses biological activity and can directly affect cell growth and differentiation (Obeid and Hannun, 1995). Short-chain C2-, C6- and C8-ceramides are widely used as cell-permeable analogs to examine effects of ceramide on intact cells (Hannun, 1994). Their use was first demonstrated in HL-60 cells, in which exposure to C_2-ceramide resulted in a dose-dependent inhibition of cell proliferation that was accompanied by induction of the monocytic phenotype (Kim *et al.,* 1991). Futhermore, C_2-ceramide induced transcriptional down-regulation of the c-myc proto-oncogene by blockade of transcriptional elongation (Kim *et al.,* 1991). These results suggest that ceramide-induced effects on cell proliferation may involve control of c-myc mRNA levels (Kim *et al.,* 1991). The effects of ceramide on cell differentiation have been extended to a number of other cell systems, such as T9 glioma cells and fibroblasts. Ceramide is also thought to play a role in the regulation of the transcription factor NFkB (Dbaibo *et al.,* 1993; Hannun and Obeid, 1995). Ceramide appears to activate NFkB poorly

when applied to intact cells, but effectively in permeabilized cells (Dbaibo *et al.,* 1993; Hannun and Obeid, 1995). This raises the possibly that ceramide may be necessary but not sufficient for activation of NFkB (Dbaibo *et al.,* 1993; Hannun and Obeid, 1995).

Early investigations of the biological activity of ceramide analogs were hindered by their potent cytotoxic effects. The cytotoxicity was stereoisomer-specific, implying that ceramide-induced cytotoxicity was likely to be the result of an interaction between ceramide and a biological receptor, rather than the result of a nonspecific detergent-like effect on membrane integrity (Saba *et al.,* 1996). In addition, molecules closely related to ceramide, such as dihydroceramide, lacked any significant cytotoxic activity (Pushkareva *et al.,* 1995). This lead to the suggestion that ceramide may be a mediator of apoptotic cell death.

Obeid and co-workers (1993) demonstrated the ability of C_2-ceramide to induce programmed cell death in U937 monoblastic leukemic cells. Ceramide potently induced internucleosomal DNA fragmentation. The effects were specific in that closely-related analogs of ceramide, such as DAG and dihydroceramide, were inactive (Jarvis *et al.*, 1994). Apoptosis can also be induced in other

cell systems using C_8-ceramide (Jarvis *et al.*, 1994). As is the case for other agonists that induce programmed cell death, the effects of ceramide can be inhibited by the addition of zinc, suggesting the involvement of calcium-dependent endonuclease (Pushkareva *et al.*, 1995). Other studies have suggested that ceramide may also participate in apoptosis induced by dexamethasome, crosslinking of antibodies, FAS ligand, or serum withdrawal (Pushkareva *et al.*, 1995). Obeid and co-workers (personal communication) have shown that transfection of a bacterial neutral SMase results in apoptosis of mammalian cells, while incubation of the cells with the exogenous bacterial SMase does not. These results suggest that despite the localization of sphingomyelin and/ or SMase to the outer leaflet of the plasma membrane, ceramide must be generated within cells to initiate apoptosis.

A role for ceramide in cell-cycle regulation has also been proposed. In MOLT-4 leukemic cells, it was shown that serum withdrawal resulted in significant accumulation of ceramide (Jayadev *et al.*, 1995). This was concurrent with the development of cell-cycle arrest in G0/G1 and initiation of apoptosis (Jayadev *et al.*, 1995). When MOLT-4 cells were treated with C_6-ceramide, a

specific arrest in the G0/G1 phase of the cell cycle was observed that was both dose- and time-dependent (Pushkareva *et al.,* 1995).

The retinoblastoma (Rb) gene product is centrally involved in regulation of the mammalian cell cycle. It inhibits cell-cycle progression, particularly in response to growth suppressors (Weinberg, 1991). The effects of ceramide on Rb phosphorylation were examined in MOLT-4 cells (Dbaibo *et al.,* 1995). The addition of C_6-ceramide, in concentrations similiar to those achieved during serum deprivation, resulted in a time- and dose-dependent dephosphorylation of Rb. Subsequent studies performed in Rb-deficient cells showed them to be resistent to ceramide-mediated growth inhibition. Cell lines that expressed either the tumor antigen SV40 or adenovirus EIA were also unresponsive to the growth-inhibitory effects of ceramide (Dbaibo *et al.,* 1995). These results indicate that ceramide may participate in an Rb-dependent pathway leading to cell cycle arrest (Pushkareva *et al.,* 1995).

Ceramide levels were found to be significantly elevated in senescent cells, which are unable to undergo DNA synthesis or proliferative responses to serum stimulation (Venable *et al.,* 1994). A role for ceramide in inducing senescence was demonstrated by its

ability to induce Rb dephosphorylation and cell cycle arrest in human diploid fibroblasts. Exogenously administered ceramide inhibited DAG generation and PLD activation in WI-38 human fibroblasts, thus mimicking the lack of PLD activation seen in senescent cells.

Ceramide is also suggested to interact with the components of other signaling pathways. It was shown that C_2-ceramide markedly enhanced the production of PGE_2 in response to stimulation of human dermal fibroblasts with IL-1 (Ballou *et al.,* 1992). Further studies reported that ceramide treatment also induced the expression of cyclooxygenase gene mRNA, and an increase in cyclooxygenase protein levels (Ballou *et al.,* 1992). Cyclooxygenase is the rate-limiting enzyme in the synthesis of PGE_2 (Ballou *et al.,* 1992).

Ceramide has also been implicated in the regulation of PLD (Venable *et al.,* 1996). In HL-60 cells, ceramide has been shown to inhibit phorbol-ester-induced PLD activation. Ceramide did not interfere with translocation of protein kinase C to the membrane in response to phorbol ester. In a cell-free HL-60 system, ceramide interfered with PKC-mediated activation of PLD.

Similiar to the effects observed in various mammalian cell lines, it has been found that growth of *Saccharomyces cerevisiae* is

markly inhibited by C_2-ceramide (Fishbein *et al.,* 1993). This effect is dose-dependent, stereospecific, and can be elicited by phytoceramide, the naturally-occuring yeast ceramide, but not by dihydroceramide (Fishbein *et al.,* 1993). Sphingosine and phytosphingosine can also inhibit yeast growth (Ella *et al.,* 1996). A ceramide-responsive phosphatase activity was identified in crude extracts of *Saccharomyces cervisiae* (Fishbein *et al.,* 1993). While a SMase with activity at acid and neutral pH has been identified in yeast, it is not yet clear whether this enzyme hydrolyzes endogenous yeast sphingolipids.

In summary, the available data indicate that ceramide serves as a lipid mediator in mammalian cells. However, the lack of information concerning the structure and regulation of neutral SMase has impeded progress in this area.

1-10. Molecular Targets of Ceramide Action

Ceramide is postulated to serve as a second messenger molecule. Several potential targets for ceramide have been identified. To exert its biological effects, ceramide may transduce intracellular signals by activating protein kinases (Mathias *et al.,*

1991; Liu *et al.,* 1994) or protein phosphatases (Drobrowsky and Hannun, 1993). Kolesnick and co-workers (Kolesnick and Golde, 1994) have described a ceramide-activated protein kinase (CAPK) in A431 human epidermal carcinoma and HL-60 cells. This enzyme appears to phosporylate the epidermal growth factor receptor and is activated by TNF-α and IL-1 (Kolesnick and Golde, 1994). Neither the specificity of ceramide activation of CAPK nor its role in intracellular signaling have been fully elucidated.

PKC-ζ is also a postulated target for ceramide action. This isozyme of PKC is not activated by DAG or phorbol esters. PKC-ζ can be activated by ceramide *in vitro* and has been shown to play a role in ceramide- and TNF-α induced activation of the NFkB transcription factor (Lozano *et al.,* 1994).

Ceramide-activated protein phosphatase (CAPP) is another target for ceramide action. This phosphatase belongs to the class 2A (PP2A) family of serine/threonine protein phosphatases (Hannun, 1994). Studies using purified and reconstituted PP2A have shown that ceramide activates only heterotrimeric PP2A, and that the presence of the B-regulatory subunit of PP2A is essential for

imparting ceramide responsiveness (Dobrowsky and Hannun, 1993; Hannun, 1994). CAPP is activated by short and long chain ceramides, but not by sphingomyelin, sphingosine, or dihydroceramide (Dobrowsky and Hannun, 1993; Hannun, 1994). The specificities of lipid mediators for CAPP activation *in vitro* closely matches their specificities for various cellular activities of ceramide, including apoptosis (Dobrowsky and Hannun, 1993; Hannun, 1994). Okadaic acid, an inhibitor of serine/threonine protein phosphatases, inhibits CAPP *in vitro* over a concentration range of 1-10 nM (Dobrowsky and Hannun, 1993). Okadaic acid has also been shown to inhibit the effects of ceramide on c-myc downregulation as well as on growth inhibition (Dobrowsky and Hannun, 1993). These results provide evidence for the involvement of CAPP in ceramide-mediated responses.

In summary, it appears that there may be several proteins serving as direct or indirect targets for the action of ceramide. To further establish the roles of PLD and SMase in signal transduction, the relationship of their activation to that of members of the mitogen-activated protein kinase (MAPK) family was explored in this thesis project. Members of the MAPK family will be discussed

below.

1-11. Historical Overview of Mitogen-Activated Protein Kinases

MAPK was first identified by Sturgill and Ray (1986) in 3T3-LI adipocyte cells. This enzyme was referred to as microtubule-associated protein kinase 2 kinase, or MAP-2 kinase, because it utilized MAP-2 as substrate *in vitro.* These researchers found that this kinase could phosphorylate and reactivate phosphatase-treated ribosomal S6 kinase (RSK) (Sturgill and Ray, 1987). RSK (pp 90^{rsk}) is a 90 KDa S6 kinase that is activated by serine/threonine phosphorylation in response to mitogens and inactivated by protein phosphatases 1 and 2A (Sturgill and Wu, 1991). Subsequent experiments confirmed the previous observations with the use of insulin-activated MAP-2 kinase and inactive RSK from rat liver (Gregory *et al.,* 1989), and epidermal growth factor stimulated MAP-2 kinase and inactive RSK from Swiss 3T3 cells (Gregory *et al.,* 1989; Ahn and Krebs, 1990). These studies implicated MAP-2 kinase as a RSK kinase (Gregory *et al.,* 1989; Ahn and Krebs, 1990). Later, two forms of MAP-2 kinase with molecular weights of 42 KDa and

44 KDa were purified from fibroblasts (Cooper, 1989). Due to the wide substrate specificity of these kinases, the original name of MAP-2 kinase was later changed to MAPK for "mitogen-activated protein kinase" (Boulton *et al.,* 1990 Sturgill and Wu, 1991). However, when the 42- and 44-KDa kinases were sequenced, the gene products were referred to as ERKs (extracellular signal-regulated kinases) (Boulton *et al.,* 1990; Sturgill and Wu, 1991). The two isoforms are referred to as ERK1 and ERK2 (Boulton *et al.,* 1990; Sturgill and Wu, 1991). Activation of these kinases requires phosphorylation on threonine and tyrosine residues. However, removal of a phosphate either from the threonine (by phosphatase 2A) or from the tyrosine (by CD45) results in inactivation of both ERK1 and ERK2 (Anderson *et al.,* 1990; Boulton *et al.,* 1991). Additional studies have confirmed the existence of several isoforms that comprise the MAPK superfamily.

1-12. Mitogen-Activated Protein Kinase Family

Mitogen-activated protein kinases (MAPKs) comprise a family of proline-directed serine/threonine protein kinases that have been implicated in the control of a broad spectrum of biological and

cellular events (Nishida and Gotoh, 1993; Blenis, 1993). MAPK members are considered components of the intracellular regulatory network that transduces extracellular cues to initiate intracellular responses (Cano and Mahadevan, 1995). These kinases are activated by separate signaling cascades conserved through evolution. Many components of the pathways are expressed in yeast, nematodes, *Drosophila,* and mammals (Cano and Mahadevan, 1995). Three major groups of MAPKs have been identified in mammalian cells. These are 1) the extracellular signal-regulated protein kinases, ERK1 and ERK2, 2) the c-Jun NH_2-terminal kinase or stress-activated protein kinase, JNK/SAPK, and 3) p38 (HOG1) kinase (Cano and Mahadevan, 1995; Osborn and Chambers, 1996). Each MAPK group has distinct upstream activators and substrates (Cano and Mahadevan, 1995).

MAPK pathways have been well characterized in yeast. Distinct MAPK cascades transduce signals that control pheromone responses, cell-wall biosynthesis, and osmosensitivity in yeast (Ammerer, 1994). Yeast MAPKs differ from mammalian MAPKs in that they regulate separate phenomena; i.e., they seem not to be activated in parallel in response to the same stimulus, and mutations in one cascade do not affect the phenomena regulated by

other stimuli (Ammerer, 1994; Blumer and Johnson, 1994). In contrast, the mammalian MAPK subtypes can be activated simultaneously via distinct parallel cascades in response to the same stimulus (Cano and Mahadevan, 1995).

1-13. Extracellular Signal-Related Protein Kinases

ERK1 ($p44^{mapk}$) and ERK2 ($p42^{mapk}$) are the predominant MAP kinase isoforms expressed in most mammalian cells (Boulton *et al.*, 1991). These kinases are structurally very similar and are widely expressed in various cells and tissues. ERK1 and ERK2 are activated by a number of extracellular stimuli, including growth factors (e.g., epidermal growth factor), cytokines, and some hormones that act through G-protein-coupled receptors (Schlessinger, 1993; Blenis, 1993; Nishida and Gotoh, 1993; Marshall, 1994). Phorbol ester tumor promoters and other activators of protein kinase C can produce dramatic stimulation of both ERK1 and ERK2 (Pelech *et al.*, 1992). ERKs are implicated in an array of cellular mitogenic events, including cell cycle progression and the control of meiosis. ERK1 and ERK2 are inactive when dephosphorylated. Maximal activation is achieved by phosphorylation of both a threonine and a tyrosine

residue (Ahn *et al.,* 1992; Zheng and Guan, 1993; Denton and Tavare, 1995). This activation is brought about by the dual-specificity MAP kinase kinases, MEK1 and MEK2 (Ahn *et al.,* 1992; Zheng and Guan, 1993; Denton and Tavare, 1995). Downstream substrates regulated by ERKs include transcription factors (e.g., Elk-1 and ATF2), protein kinases (e.g., $p90^{rsk}$), and several other targets (Davis, 1993).

p54 MAPK and $p44^{mpk}$ are other isoforms of MAPK that have been isolated, but characterized to a lesser degree than ERK1 and ERK2. p54 MAPK is found to be stimulated in the livers of cycloheximide-treated rats (Pelech and Sanghera, 1992). This isoform is approximately 50% homologous to rat ERK2 within its catalytic domain (Pelech and Sanghera, 1992). The p54 MAPK phosphorylates MAP2 preferentially, and is sensitive to inhibition by protein-tyrosine phosphatases as well as by the serine/threonine phosphatase 2A (Seger and Krebs, 1995).

$p44^{mpk}$ (MBP kinase) is a MAPK isoform that was originally shown to be activated near the onset of germinal vesical breakdown in maturing sea star oocytes (Pelech and Sanghera, 1992). Protein and nucleic acid sequencing studies have shown that $p44^{mpk}$ shares about 77% amino acid identity with ERK2 and ERK1 (Pelech and

Sanghera, 1992). p44^{mpk} is activated *in vitro* by the src-family kinase p56^{lck} (Pelech and Sanghera, 1992). Purified p44^{mpk} can readily autophosphorylate on a serine residue with no detectable tyrosine phosphorylation (Sanghera *et al.,* 1991). However, tyrosine phosphorylation occurs during oocyte maturation and is required for activation (Sanghera *et al.*, 1991).

1-14. c-JUN NH_2-terminal Kinases

JNKs, also termed stress-activated protein kinases (SAPKs), comprise a subfamily of MAPKs that bind to and phosphorylate the transcription factor c-Jun within its NH_2-terminal domain (Hibi *et al.,* 1993). JNKs also phosphorylate other transcription factors, such as ATF2 and Elk-1, and thereby augment their transcriptional activity (Derijard *et al.,* 1994). These kinases have been characterized in a variety of mammalian cells and tissues. Two major forms of JNK, JNK1 (46KDa) and JNK2 (55KDa), have been characterized in human cells (Hibi *et al.,* 1993). The 46-KDa (JNK1) and 55-KDa (JNK2) kinases are similarly regulated. Both phosphorylate the amino-terminal domains of c-Jun, thereby potentiating its trans-activation function (Hibi *et al.,* 1993). Both

forms require phosphorylation at threonine and tyrosine residues by MAP kinase kinase 4 (MKK4/SEK1), a dual specificity kinase (Sanchez *et al.*, 1994; Davis, 1995; Derijard *et al.,* 1995). JNKs are activated by a number of environmental stress-inducing stimuli; such as UV light (Derijard *et al.,* 1994), gamma radiation (Chen *et al.,* 1996), protein synthesis inhibitors (Kyriakis *et al.,* 1994), ceramide (Westwick *et al.,* 1995), DNA-damaging drugs (Yu *et al.,* 1996b), chemopreventative drugs (Yu *et al.*, 1996a), and inflammatory cytokines (Kyriakis *et al.,* 1994). In addition, JNK activity can also be induced by mitogenic signals, including growth factors, oncogenic Ras, CD40 ligation, and T-cell co-stimulation (Derijard *et al.,* 1994; Minden *et al.,* 1994; Su *et al.,* 1994; Sakata *et al.,* 1995; Berberich *et al.,* 1996; Chen *et al.,* 1996). In T-cells, JNKs can be synergistically activated by treatment with phorbol ester and calcium ionophore (Su *et al.,* 1994). JNKs are implicated in both cell proliferation and apoptotic cell death.

1-15. p38 MAP Kinases

p38 MAPK is a homolog of the yeast HOG1 (high-osmolarity glycerol response-1) kinase (Derijard *et al.,* 1995). p38 is activated

by dual phosphorylation on tyrosine and threonine residues by MKK3 and MKK6 (Derijard *et al.,* 1995; Han *et al.,* 1996). Activation of p38 MAPK is induced in response to lipopolysaccharide, hyperosmolarity interleukin-1, endotoxin, TNF, pro-inflammatory cytokines, heat shock, and DNA-damaging agents (Pandey *et al.,* 1996). Activation of p38 MAPK can occur through c-Abl protein tyrosine kinase-dependent and -independent mechanisms (Pandey *et al.,* 1996). The substrate specificity of p38 MAPK differs from ERK or JNK kinases. p38 MAPK strongly phosphorylates transcription factor ATF2 (Post and Brown, 1996), but does not phosphorylate $cPLA_2$, c-Myc, or c-Jun (Raingeaud *et al.,* 1995). p38 MAPK appears to play a role in regulating inflammatory responses, including cytokine secretion and apoptosis (Xia *et al.,* 1995).

1.16. MAPK Phosphorylation Cascades

Activations of MAPKs occur through a series of sequential protein-protein interactions and phosphorylation reactions (Seger and Krebs, 1995). Each MAPK cascade consists of a module of three kinases: a MAPK kinase kinase (MEKK or MKK), which phosphorylates and activates a dual-specificity MAPK kinase (MEK), which in turn

phosphorylates and activate a MAPK (Cobb and Goldsmith, 1995). Members of the G-protein Ras superfamily are involved in the activation of MAPK phosphorylation cascades (Marshall, 1995; Davis, 1993). In the case of the ERK cascade, Ras exchanges GDP (guanosine diphosphate) for GTP (guanosine triphosphate) upon activation of a Ras-specific guanine-nucleotide exchange factor (Viciana and Downward, 1993; Vojtek and Cooper, 1995). In the GTP-bound state, Ras then physically associates with the N-terminal region of Raf, thereby recruiting this serine/threonine kinase to the plasma membrane, where it becomes activated by an unknown mechanism (McCormick, 1994). Raf in turn activates and phosphorylates MAPK kinase (MEK), which ultimately leads to the activation of MAPK (ERK) (Crews *et al.,* 1993). Ras activation can also occur via protein-tyrosine kinase receptors and G-protein-linked receptors. In this case, autophosphorylation of the receptor protein-tyrosine kinases, occuring as a consequence of ligand binding, provides docking sites for Grb2. Grb2 in turns interacts with the nucleotide exchange factor mSOS to activate Ras (Davis, 1993; Marshall, 1995) (Figure 1-3). Activation of ERKs via G-protein-coupled receptors appears to involve c-Src and Shc (Torres and Ye, 1996).

Two member of the Rho family, Cdc42 and Rac, activate members of the JNK phosphorylation cascade in the same way that Ras activates Raf (Cano and Mahadevan, 1995; Vojtek and Cooper,1995; Coso *et al.,* 1995). It is believed that the activation of MEKK by Cdc42 and Rac is mediated by PAK65 (Vojtek and Cooper, 1995). PAK65 is a Cdc42- and Rac-binding protein (Vojtek and Cooper, 1995). The direct binding of PAK65 to Cdc42 and Rac requires active GTP-bound Cdc42 or Rac, and stimulates the catalytic activity of MEKK, which then phosphorylates and activates JNK kinase (SEK), which in turn phosphorylates and activates JNK/SAPK (Figure 1-3) (Vojtek and Cooper, 1995).

The biological consequences and mechanism of activation of the mammalian p38 MAPKs are poorly understood and are under intense investigation. These kinases are postulated to be regulated similarly to the yeast HOG1 phosphorylation cascade. A MAP kinase kinase kinase (MAPKKK) referred to as apoptosis signal-regulating kinase 1 (ASK1) can activate MKK3/MAPKK6, which in turn activates the mammalian p38 MAPK and JNK (Ichijo *et al.,* 1997) (Figure 1-3).

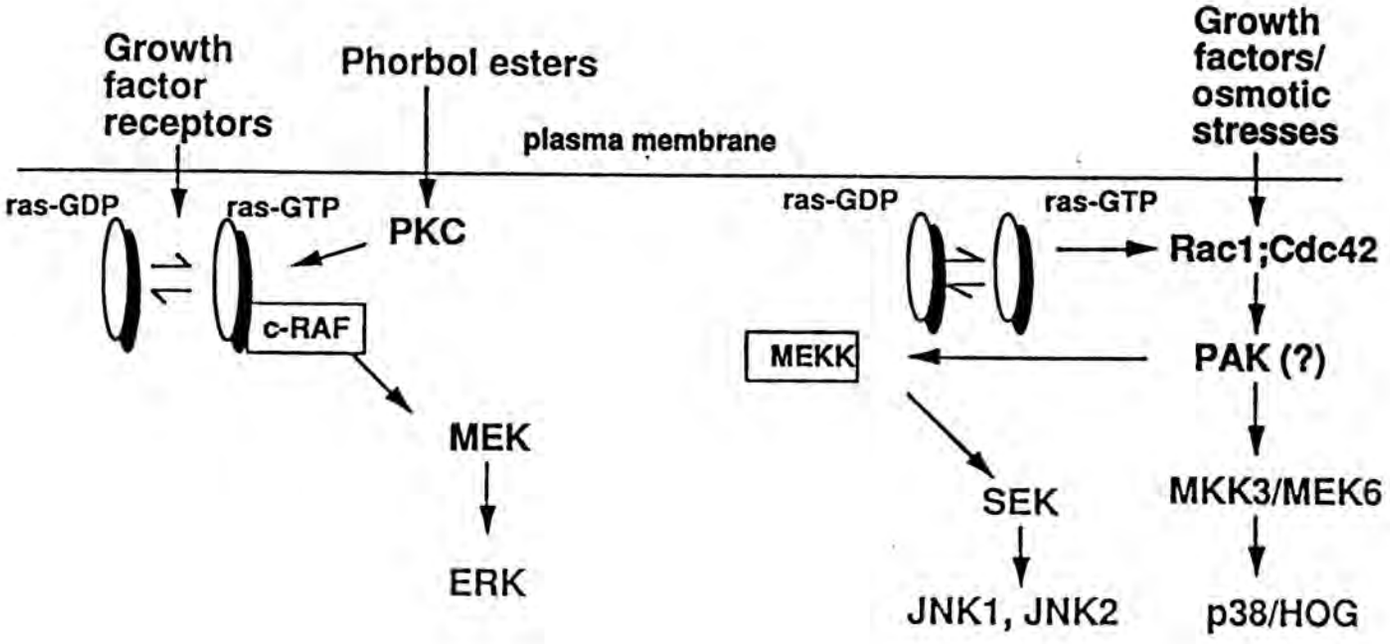

FIGURE 1-3. Mitogen-activated protein kinase signaling pathways.

ERK, JNK, and p38 are MAP kinase family members that are activated through parallel kinase cascades. Interactions between components of the various pathways may occur. ERKs are activated by multiple G protein-linked receptors, growth factors, and phorbol esters. JNKs are activated by growth factors and stress-related responses. p38 MAPK is also regulated by stressful stimuli. Activation of the kinases ultimately leads to the induction of various transcription factors.

1-17. Goals and Significance of Thesis Project

The overall goal of this thesis project was to investigate the roles of PLD and SMase in signal transduction in T-lymphocytes. Previous and concurrent work in this laboratory used A7r5 vascular smooth muscle cells and Rat-1 fibroblasts to study the potential role of agonist-induced PLD activation in ERK activation (Jones *et al.,* 1994; Knoepp et al., 1996). This laboratory also concurrently studied the expression and regulation of PLD in yeast *Saccharomyces cerevisiae* (Ella et al., 1995). Results from these studies showed that the activation of PLD is not restricted to mitogenic signaling pathways, and that PLD activation is not sufficient for ERK activation. Nonetheless, phosphatidic acid has been proposed to regulate Raf (Ghosh *et al.,* 1996), and can induce proliferation in some mammalian cell types (Flores *et al.,* 1996). With regard to SMase, while ceramide appears to be involved in induction of apoptosis, products of sphingomyelin metabolism can also induce proliferation. Ceramide can activate ERKs in some cells (Olivera *et al,* 1992) and JNKs in others (Westwick *et al.,* 1995). Thus, the role of PLD and SMase require further clarifications.

It is now apparent that MAPKs can play very different roles in

different cell types. This is particulary apparent when fibroblasts and T-lymphocytes are compared. ERK activation is required for mitogenesis in fibroblasts, but can be involved in cell death in T-cells (Sansbury *et al.,* submitted). JNK activation is required for proliferation of T-cells (Su *et al.,* 1994), but is involved in apoptosis in fibroblasts. Since the regulation of T-lymphocyte proliferation and survival is of great importance with respect to human health, the roles of lipid mediators in regulating MAPK activities in T-cells is the focus of this thesis project.

Two T-lymphocyte cell lines, Jurkat and EL4, were used in this project. These are IL-2 independent human and murine cell lines, repectively. Jurkat has been extensively used as a model to study early events in T-lymphocyte activation and signal transduction. EL4 has been used for many years to study signal transduction leading to IL-2 expression.

When this project was initiated, very little was known about the roles of PLD, SMase, and MAPKs, in T-cells. Products of the PLD and/or SMase reaction were proposed to participate in the activation of MAPKs, JNKs, or other protein kinases. Both ERKs and JNKs are activated in T-cells in response to ligation of the T-cell receptor.

We therefore hypothesized that PLD and SMase are involved in MAPK activation in T-lymphocytes.

To address this hypothesis, Specific Aim I of this thesis project characterizes the effects of phorbol ester (PMA), and the antileukemic drug (Ara-C), on PLD and SMase activity in T-cells. Reports from other laboratories had shown that Ara-C activates SMase in some lymphoid cells (Strum *et al.,* 1994). We show that treatment with phorbol ester stimulates acute activation of PLD in Jurkat, but does not affect SMase activity in either EL4 or Jurkat cells. Ara-C does not affect PLD activity in either cell line, but increases SMase activity in EL4 and Jurkat T-cells. EL4 cells appear to lack PMA-stimulated PLD activity.

Specific Aim II of this thesis project explores the role of PLD and SMase in activation of ERKs and JNKs in T-cells. Activation of ERKs is detected in EL4 and Jurkat T-cells in response to treatment with phorbol esters. This activation does not however, appear to depend on PLD activation. Moreover, ERK activation is unaffected by treatment with Ara-C or ceramides. JNK activity is synergistically increased in EL4 and Jurkat following treatment with phorbol esters and calcium. Ara-C induces delayed activation of JNKs in both EL4

and Jurkat cells.

Specific Aim III of this thesis project examines mechanisms underlying the activation of PLD and SMase. We conclude that PMA-induced activation of PLD is probably not due to translocation of PLD or a direct interaction of PKCα with PLD

Our findings from this project suggest that PLD and SMase are independently activated, and represent distinct signaling pathways in T-cells. The results of this study contribute to our understanding of T-cell activation and death. Moreover, the information obtained from this study provides additional knowledge regarding basic mechanisms involved in the activation of PLD, SMase, ERKs, and JNKs in T- lymphocytes.

CHAPTER 2

Effects of Phorbol Ester on Phospholipase D and Mitogen-Activated Protein Kinase Activities in T-Lymphocyte Cell Lines

2-1. Introduction

Activation of both phospholipase D (PLD) and ERK mitogen-activated protein kinases are generally seen in response to phorbol ester in non-lymphoid cells. PLD hydrolyzes phosphatidylcholine to phosphatidic acid (PA), which can be converted to diglyceride and other lipid mediators (Liscovitch, 1991). ERKs, which are activated via a ras/Raf-dependent pathway, generally mediate mitogenic signals (Seger and Krebs, 1995). The c-Jun N-terminal kinases (JNKs) phosphorylate and activate c-Jun (Kyriakis *et al.,* 1994). JNKs, which are synergistically activated in T-cells by phorbol ester and calcium, have been linked to transcription of interleukin-2 (IL-2) (Su *et al.,* 1994). Both ERKs and JNKs are activated in T-cells following ligation of the T-cell receptor (TCR) (Su *et al.,* 1994).

The mechanism of activation of PLD and its role in cellular signalling remain to be established. At least two forms of mammalian PLD exist (Hammond *et al.,* 1995; Morris *et al.,* 1996). Some PLDs can be activated by the GTP-binding proteins ADP-ribosylation factor (ARF) (Brown *et al.,* 1993; Cockcroft *et al.,* 1994; Massenburg *et al.,* 1994; Hammond *et al.,* 1995) or rho (Malcolm *et al.,* 1994; Lambeth *et al.,* 1995). Calcium-dependent activation of

PLD has also been reported (Olson *et al.,* 1991; Gustavsson *et al.,* 1994). Primary T-lymphocytes and most T-cell lines do not exhibit phorbol ester-activated PLD activity (Kinsky *et al.,* 1989). However, in the Jurkat human leukemic cell line, PLD is activated by phorbol ester or TCR ligation (Stewart *et al.,* 1991). Membrane PLD activity is present in mouse thymus, but not in primary murine or human T cells (Ella *et al.,* 1994; Meier *et al.,* 1994). These observations suggest that expression of PLD activity is regulated during T-cell differentiation and transformation.

Activation of PLD has been proposed to play a role in mitogenic signalling (Kondo *et al.,* 1992). PLD can be activated by growth factors in a pathway downstream of PI-PLC and PKC (Lee *et al.,* 1994). PA can be converted to diacylglycerol by PA phosphohydrolase, potentially supporting continued PKC activity. PA itself may act as a second messenger. A role for PA in Raf activation has been proposed (Ghosh *et al.,* 1996). The role of PA in mitogenic responses is likely to vary between cell types (Jones *et al.,* 1994). The goal of this study was to examine the effects of phorbol ester on PLD, ERK, and JNK activities in the Jurkat and EL4 T-lymphocyte cell lines.

2-2. Materials and Methods

Cell Culture

EL4 cell lines were originally provided by Dr. David Morris (Univ. of Washington); phorbol ester-responsive wild-type (WT) cells were used unless otherwise noted. Phorbol ester-resistant variant EL4 cells, in which PMA does not activate MAPKs, (Meier *et al.*, 1991) were used for some experiments. Jurkat and HL-60 cells were obtained from Drs. Sandra Slivka (Tanabe Research Laboratories) and Khapil Bhalla (MUSC), respectively. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (UBI), non-essential amino acids, and penicillin/streptomycin.

Phospholipase D Assays

For PLD assays in intact cells, approximately $1x10^6$ cells were incubated overnight in complete culture medium (1 ml) containing 10 μCi/ml [^{3}H]-palmitic acid (Dupont NEN). Triplicate or quadruplicate samples were used for each experimental condition. Cells were washed with ice-cold PBS and collected by low-speed centrifugation (Beckman). The cells were then incubated with and without 100 nM

PMA (LC Laboratories) in the presence and absence of 0.5% ethanol. Ethanol was added immediately prior to addition of PMA. At the conclusion of the incubation, cells were washed and ice-cold methanol/6 N HCL (50:2 v/v, 0.3 ml) was added. This mixture was transferred to a glass tube, and 0.5 ml chloroform was then added. Phase separation was accomplished by the addition of 0.3 ml of 1M NaCl. The lower organic phase was recovered and dried under nitrogen gas. Cellular lipids were extracted from the cells with 30 µl chloroform/methanol (90:10 v/v). Dipalmitoyl-PA (Sigma) and phosphatidylethanol (PEt) (Avanti Polar Lipids Corporation) were added to each sample as chromatography standards. Phospholipids were separated using thin-layer chromatography developed with a solvent mixture consisting of the upper phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (90:50:20:100, v/v in ml) to which 1 ml acetic acid was added. Areas containing PA and PEt, as well as the remainder of the lanes, were scraped and transferred to vials for liquid scintillation spectrometry (Beckman). The radioactivity contained in the PA and PEt bands is expressed as a percent of the total radioactivity recovered from each lane.

For PLD assays in broken cell preparations, cells were

incubated in the absence and presence of 100 nM PMA or 10 U/ml PLD from *Streptomyces* (Sigma). Cytosolic and membrane extracts were prepared as described for the ERK assay (see below). Membrane PLD activity was assessed *in vitro* using BODIPY-phosphatidylcholine (BPC; 2-decanoyl-1-(0-(11-(4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza,s-indacene-3-propionyl)amino)undecyl)sn-glycero-3-phospho-choline; from Molecular Probes), as the fluorescent substrate. The reaction, containing 0.1 mM BPC, 150 mM NaCl, 200 μM octylglucoside, 25 mM MES (pH 6.0), 10 mM HEPES (pH 7.5), %mM EGTA, 1mM EDTA, 40 mM β-glycerophosphate (pH 7.5), 1mM DTT, and 1% n-butanol in a final volume of 12.5 μl, was initiated by addition of 10 μg of membrane protein and incubated for 30 minutes at 30°C. Cabbage PLD, used as a positive control, was from Sigma. A 5-μl aliquot of the reaction mixture was applied to a plastic-backed silica gel G60 TLC plate without fluorescent indicator (Merck). The plate was developed using chloroform/methanol/water/acetic acid (45:45:10:1, v/v) Results were imaged using a FluorImager (Molecular Dynamics) or GDS 5000 gel documentation and analysis system (UVP Life Sciences). For some experiments, results were

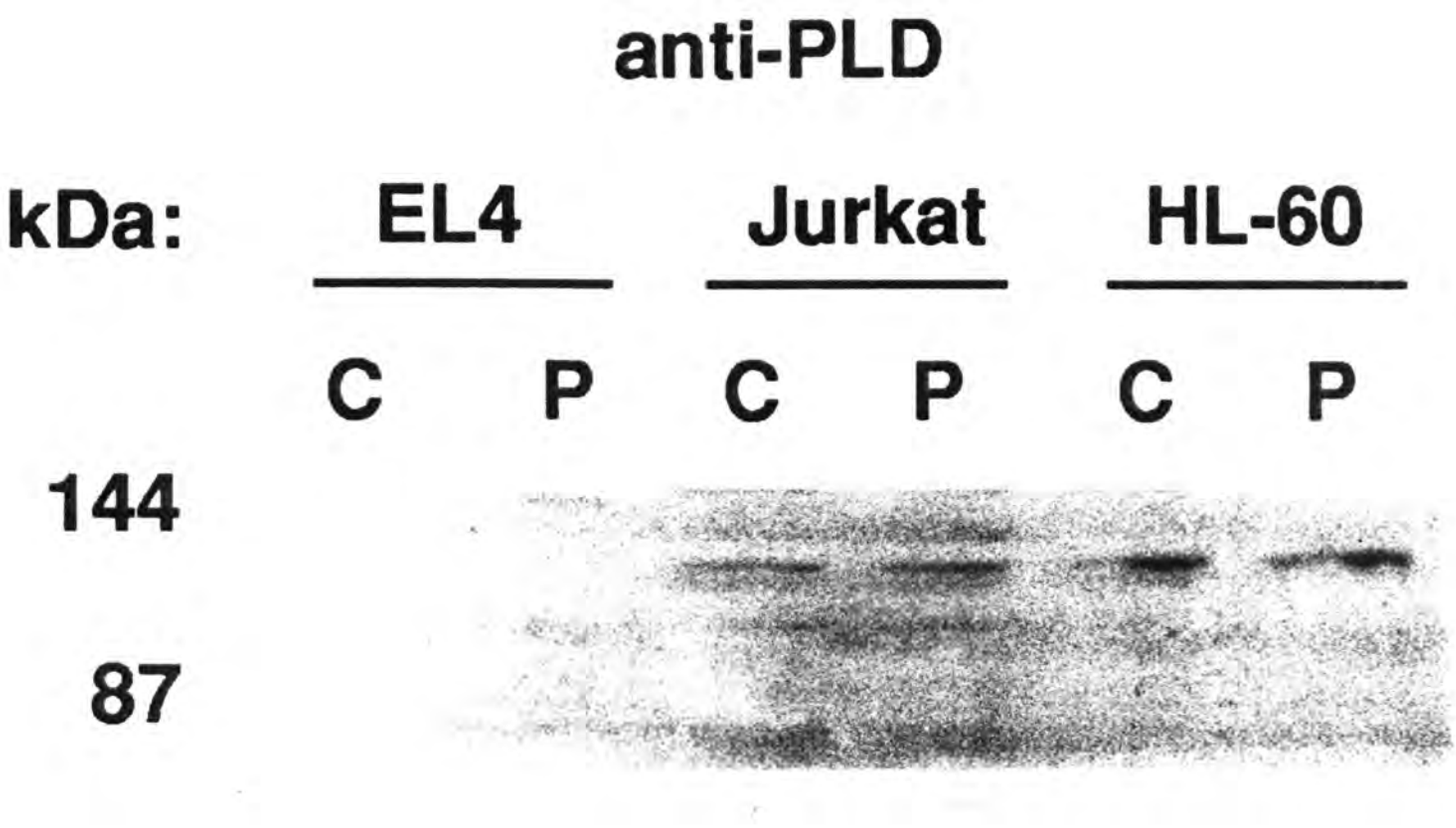

FIGURE 2-5. Immunoblots for PLD in EL4, Jurkat, and HL-60 cells.

Equal amounts of membrane protein from EL4 and Jurkat cells were separated by SDS-PAGE and then immunoblotted with anti-PLD antibody. The migration positions of molecular size markers are shown on the right.

recognized a 120-kDa protein in Jurkat and HL-60. A doublet was visible is some experiments. The immunoreactive bands were predominantely localized to the membrane fraction (Figure 2-6). The mobility and localization of these bands were not altered by PMA treatment in Jurkat or HL-60. These bands were not detected in EL4.

Effects of PMA on ERK Activity

We have previously shown that ERKs are activated in response to PMA in WT, but not variant, EL4 cells (Meier *et al.,* 1991). A similar response has been reported for Jurkat cells (Nel *et al.,* 1990; Whitehurst *et al.,* 1992; Marklund *et al.,* 1993; Su *et al.,* 1994). The results shown in Figure 2-7 confirm that PMA stimulates ERK activity, measured as MBP kinase activity, in both WT EL4 and Jurkat cells. MBP kinase activity was increased by 270 ± 52% in EL4, and by 181 ± 24% in Jurkat (mean ± S.E.M., n = 5 experiments for each cell line), in response to PMA. The lower fold activation in Jurkat cells likely reflects the higher basal activity of MBP kinases other than ERK in these cells; since MBP kinase activity in untreated cells was 42% higher for Jurkat than for EL4 when measured in parallel in

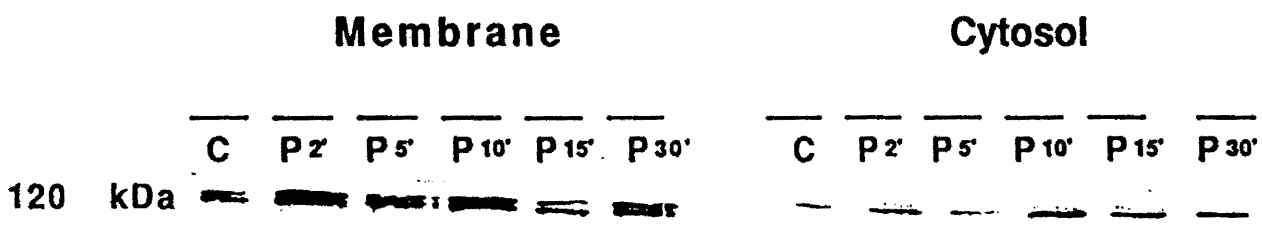

FIGURE 2-6. Immunoblot for PLD in membrane and cytosol from Jurkat cells.

Jurkat cells were incubated with or without 100 nM PMA for the indicated times. Equal amounts of membrane and cytosolic protein were separated by SDS-PAGE and then immunoblotted with anti-PLD antibody.

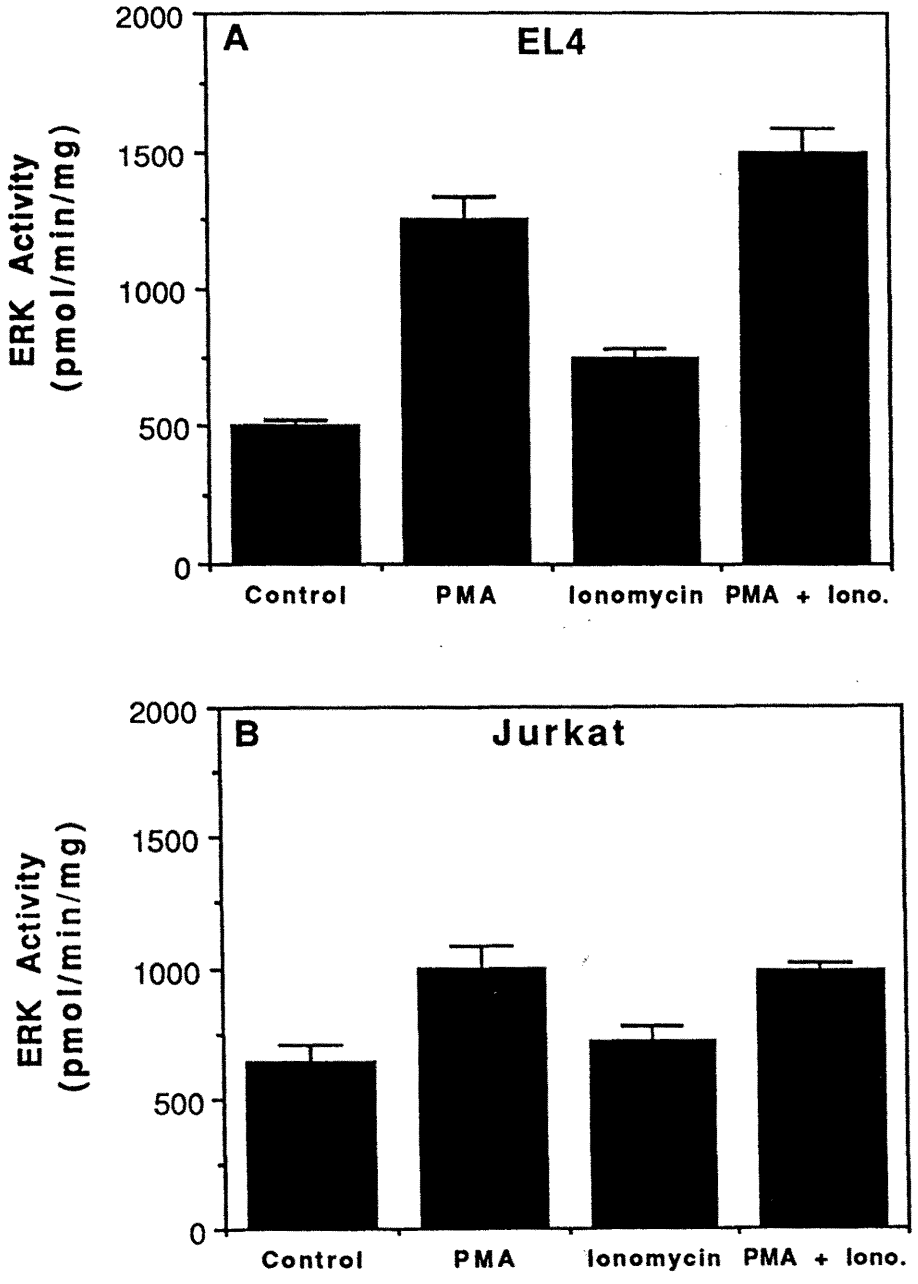

FIGURE 2-7. Effects of phorbol ester on ERK activity in Jurkat and EL4 cells.

WT EL4 (panel A) and Jurkat (panel B) cells were incubated for 10 minutes in the absence and presence of 100 nM PMA and/or 2 μM ionomycin. ERK activity was assessed in cytosolic extracts prepared from cells, using MBP as substrate. Each data point represents the mean ± S.D. of values obtained from duplicate samples of cells.

the same experiment (n = 4). A combination of PMA and 2 μM ionomycin did not produce a further increase in ERK activity in either cell line. Phosphorylation of ERKs is accompanied by a shift in their electrophoretic mobility. A major immunoreactive band of approximately 42 kDa (ERK2) was detected in both EL4 and Jurkat cells (Figure 2-8). A 44 kDa band (ERK1) was also present, but is not easily visualized in this particular immunoblot. PMA treatment resulted in a shift in the electrophoretic mobilities of ERK1 and ERK2 in both EL4 and Jurkat cells. These data indicate that PMA can induce phosphorylation of ERKs in both EL4 and Jurkat cells.

Effects of Bacterial PLD on EL4 Cells

The data presented above suggest that generation of PA is not required for ERK activation in T-cells. Incubation of A7r5 vascular smooth muscle cells with PLD from *Streptomyces* can increase membrane PLD activity and elevate PA levels (Kondo *et al.,* 1992; Jones *et al.,* 1994). While the chemical composition and localization of the PA generated has not been described, we have shown that bacterial PLD remains associated with cell membranes in A7r5 cells (Jones *et al.,* 1994). Phosphatidylbutanol (PBt) was generated by

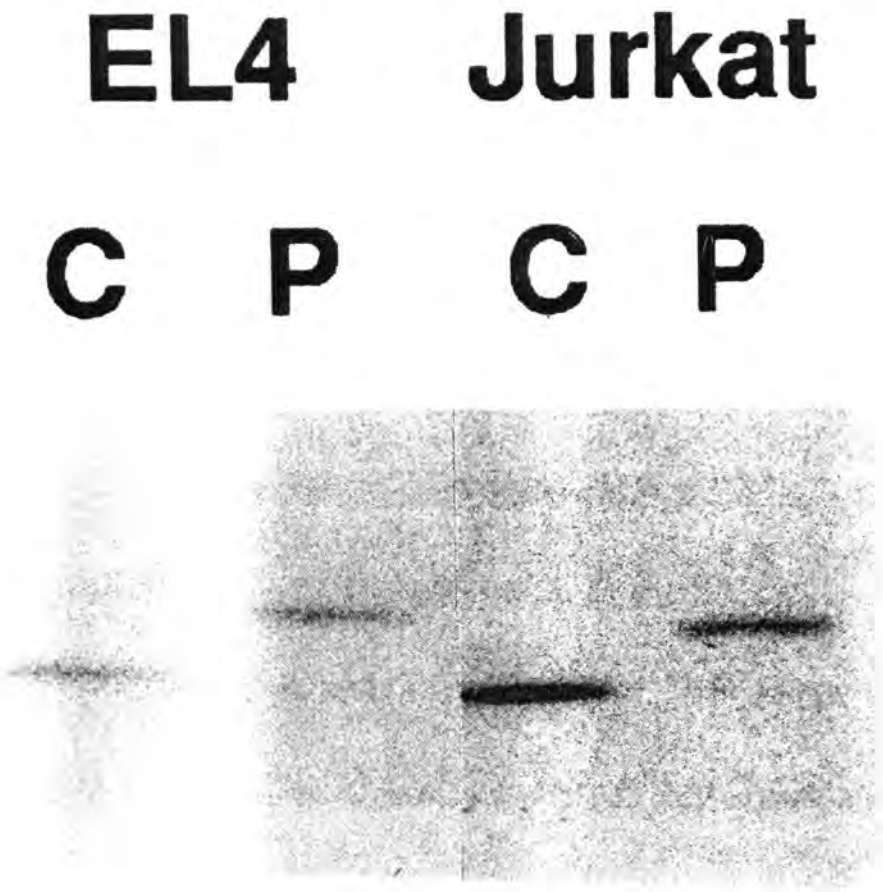

FIGURE 2-8. Immunoblot for ERK in EL4 and Jurkat cells. WT EL4 and Jurkat cells were incubated in the absence or presence of 100 nM PMA for 10 minutes. Whole cell extracts were separated on SDS-PAGE and then subjected to immunoblotting for ERKs using pan-ERK antibody. Equal amounts of cellular protein were loaded in each lane.

membranes prepared from WT and variant EL4 cells incubated with bacterial PLD (Figure 2-9). The enhanced PLD activity is presumably due to association of bacterial PLD with cell membranes. The results shown here also confirm that neither WT nor variant EL4 cells have PMA-stimulated PLD activity.

The effects of bacterial PLD on intact EL4 cells were examined. Treatment of WT EL4 cells with bacterial PLD resulted in a much greater elevation of PA than seen under the same conditions for A7r5 cells (Jones *et al.,* 1994), suggesting that EL4 is less able than A7r5 to metabolize PLD reaction products. However, while incubation with bacterial PLD resulted in profound increases in PEt and PA (Figure 2-10A), ERKs were not activated (Figure 2-10B). These data indicate that elevation of PA is not sufficient to induce activation of ERKs in EL4 cells.

Effects of PMA on JNK Activity in EL4 and Jurkat Cells

In T-cells, JNKs have been shown to be synergistically activated in response to co-stimulation with PMA and calcium ionophore (Su *et al.,* 1994). The mechanism underlying this co-stimulation has not been delineated. PMA alone has variable effects

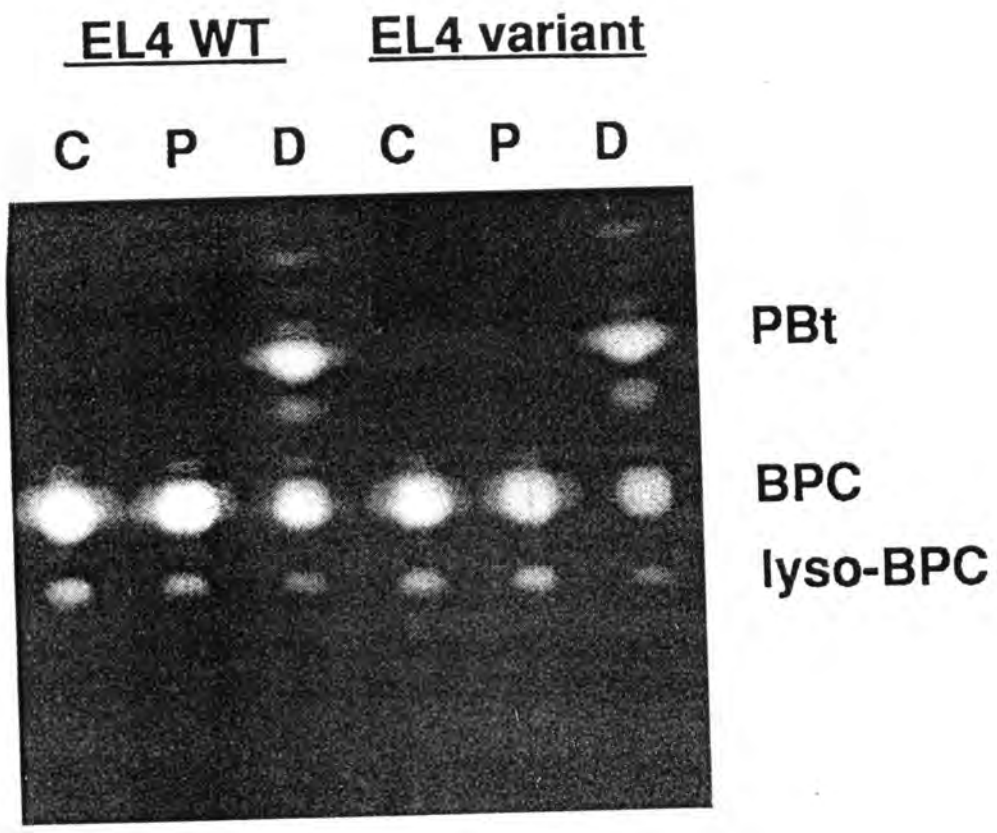

Figure 2-9. PLD Activity in EL4 Membranes

WT and variant EL4 cells were incubated with no additions ("C"), with 100 nM PMA ("P"), or with 10 U/ml bacterial PLD ("D") for 10 minutes. PLD activity was assayed in membrane extracts prepared from the cells, using the *in vitro* fluorescent assay. The TLC separation of the reaction products is shown, with the migration positions of the substrate (BPC), PA, lyso-BPC, and phosphatidylbutanol (PBt) indicated.

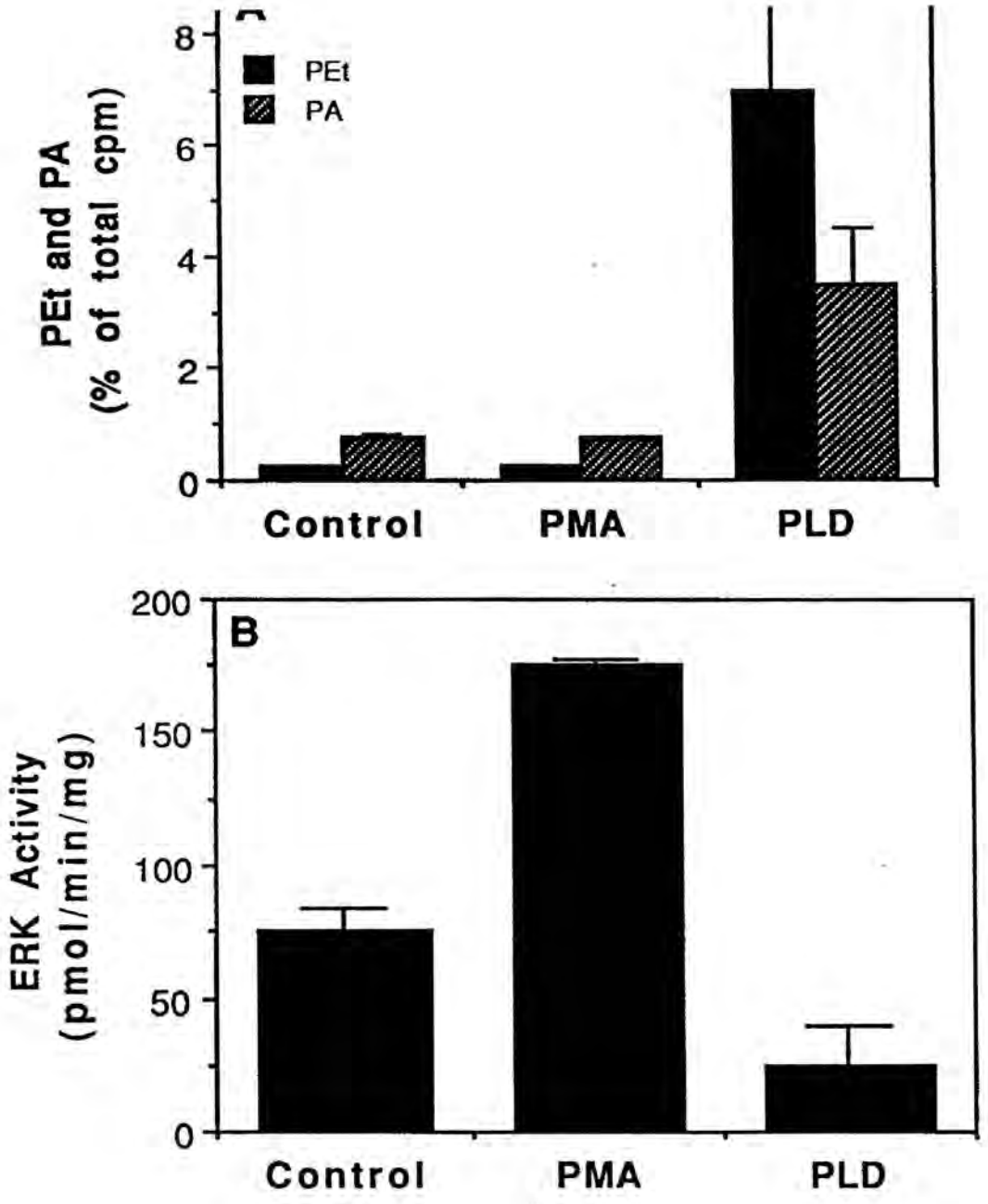

FIGURE 2-10. Effects of bacterial PLD on ERK activity in EL4 cells.

In panel A, intact WT EL4 cells were incubated with 100 nM PMA or 10 U/ml bacterial PLD for 30 minutes in the presence of 0.5% ethanol. Phosphatidylethanol (PEt) and phosphatidic acid (PA) formation was assessed as described in the text. Each data point represents the mean ± S.D. of values obtained from triplicate samples of cells. In panel B, WT EL4 cells were incubated with 100 nM PMA or 10U/ml bacterial PLD for 10 minutes. ERK activity was assessed in cytosolic extracts prepared from cells, using MBP as substrate. Each data point represents the mean ± S.D. of values obtained from duplicate samples of cells.

on JNK activity in different cell lines, with a slight activation previously reported for Jurkat cells (Su *et al.,* 1994). As shown in Figure 2-11, PMA alone caused a slight activation of JNK in both Jurkat and EL4 cells. In both cell lines, JNK was synergistically activated in response to a combination of PMA and ionomycin. Treatment of Jurkat cells with exogenous bacterial PLD did not activate JNK (Figure 2-12). Thus, activation of PLD does not appear to be required for JNK activation.

2.4. Discussion

Phosphatidic acid, generated by the PLD reaction, has been proposed to play a role in mitogenic signalling in mammalian cells. Possible mechanisms for this effect include a direct effect of PA on effector proteins (e.g., kinases or G-proteins), metabolism of PA to DG (a PKC activator), or metabolism of PA to LPA. LPA, a lipid mediator binding to a G-protein-coupled receptor, is mitogenic for some cell types (Moolenaar, 1995).

Proteins involved in the ERK activation cascade are proposed targets for PA. While activation of ERKs is an early event in mitogenic signalling, it is not necessarily sufficient to induce

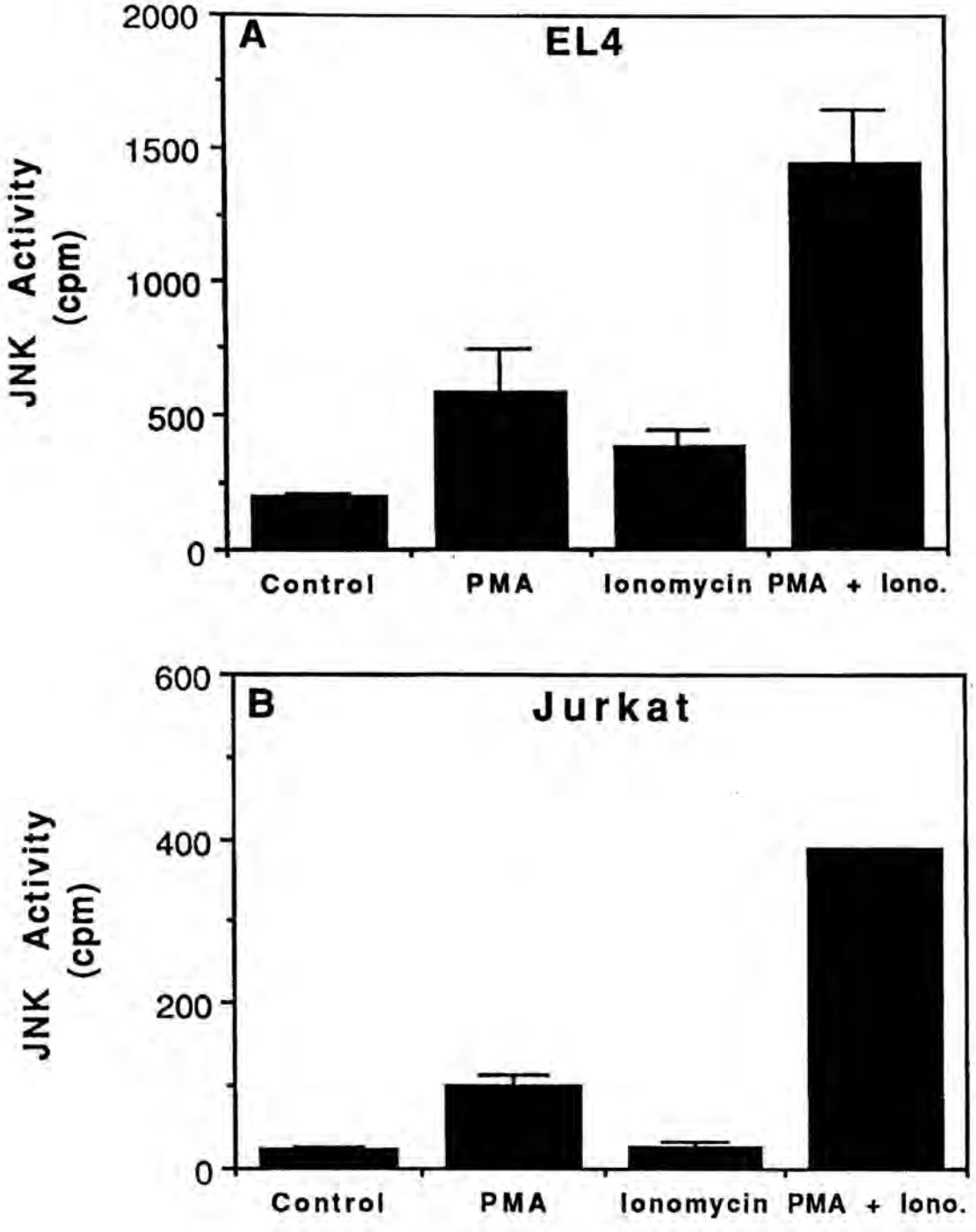

FIGURE 2-11. Effects of PMA and ionomycin on JNK activity in EL4 and Jurkat cells.

WT EL4 (upper panel) and Jurkat (lower panel) cells were incubated in the absence and presence of 100 nM PMA and/or 2 μM ionomycin for 10 minutes. JNK activity was assessed in whole cell extracts, using a solid-phase assay with GST-jun as substrate. Each data point represents the mean ± S.D. of values obtained from duplicate (Jurkat) or triplicate (EL4) samples of cells.

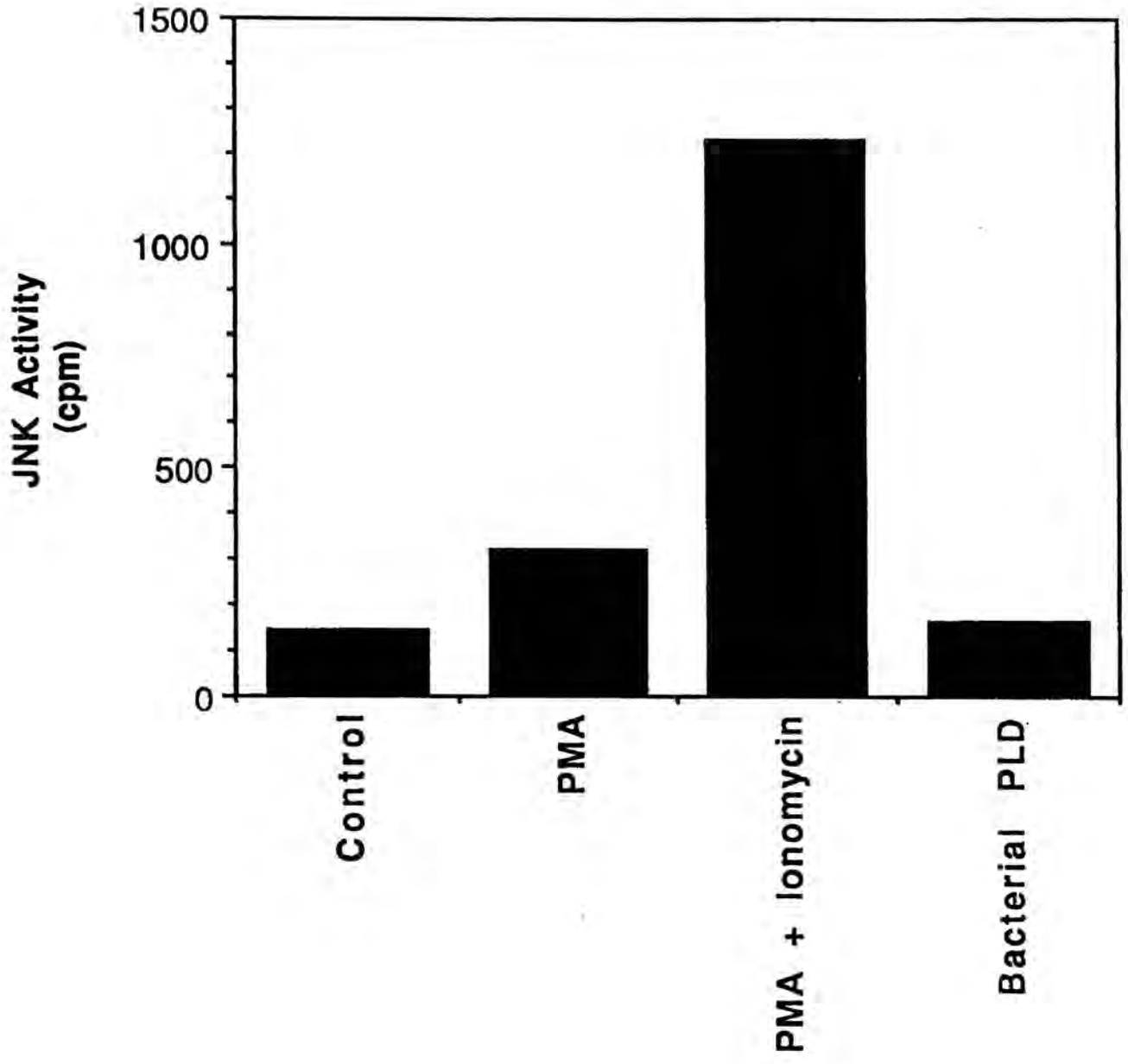

FIGURE 2-12. Effects of bacterial PLD on JNK activity in Jurkat.

Single aliquots of Jurkat cells were incubated in the absence and presence of 100nM PMA, 2 μM ionomycin, and 10 U/ml bacterial PLD for 10 minutes. JNK activity was assessed in whole cell extracts, using a solid-phase assay with GST-jun as substrate. Similar results were obtained in two separate experiments.

proliferation (Jones *et al.,* 1994; Lubinus *et al.,* 1994). In mature T-lymphocytes, ERKs are activated both by TCR ligation and by IL-2, but this activation is not required for proliferation (Alberola-Ila *et al.,* 1995). Activation of JNK, which requires co-stimulation with PMA and calcium, is necessary for induction of IL-2 synthesis (Su *et al.,* 1994).

The results of our study confirm that PLD is expressed, and is activated in response to phorbol ester, in the Jurkat cell line. However, PLD activation and PLD activity were not observed in EL4 cells. PLD activity was likewise not observed in human peripheral T-cells, murine splenic T-cells, and a variety of murine T-cell lines (Meier *et al.,* 1994; Ella and Meier, unpublished data). These results are consistent with an earlier report that PLD activity is not present in human peripheral T-lymphocytes (Kinsky *et al.,* 1989). However, substantial PLD activity is present in membranes prepared from mouse thymus (Ella *et al.,* 1994). Taken together, these results suggest that expression of PLD activity ceases at some point during T-cell differentiation.

The number of isoforms of PLD expressed in mammalian cells is unknown, with a 120-kDa form the first to be characterized at the

molecular level (Hammond *et al.,* 1995). A 120-kDa protein recognized by an antibody to PLD was expressed in Jurkat and HL-60, but not EL4. These results suggest that the absence of PLD activity in EL4 could be due to lack of expression of PLD. However, since the existence of at least one more mammalian isoform of PLD has been reported (Hammond *et al.,* 1995, Hammond *et al.,* 1997), it is possible that the other forms are expressed in T-cells, and that the 120-kDa enzyme is not responsible for PMA-stimulated PLD activity in Jurkat and HL-60. Alternatively, since the factors regulating PLD activity have not been completely defined, the lack of PLD activity in T-cells could be due to an absence of positive regulation.

The lack of correlation between PLD activity and ERK activity is reminiscent of results obtained previously for the A7r5 vascular smooth muscle cell line (Jones *et al.,* 1994). It remains possible, as proposed (Ghosh *et al.,* 1996), that PA generated by PLD can contribute to Raf activation. However, since ERKs are activated to similiar extents in both EL4 and Jurkat, any contribution of PLD to ERK activation in Jurkat cells is apparently minor.

We report here that PMA-stimulated JNK activity can be detected in either the absence or presence of PMA-stimulated PLD

activity. The ability of PMA to synergistically activate JNK in EL4 cells, in which PLD is not activated, argues against a role for PLD in JNK activation. The observation that co-stimulation with PMA and ionomycin induces synergistic activation of JNK, but not PLD, suggests that JNKs do not play a role in PLD activation. It remains possible that activation of PLD in response to a physiologic agonist could contribute to other aspects of T-cell co-stimulation. For example, in some cell types, PA may serve as a source of DG to sustain PKC activation (Martinson *et al.,* 1990). PA can also serve as a substrate for the PLA_2-mediated production of lyso-PA (LPA) (Moolenaar 1995). LPA is mitogenic for fibroblasts (Van Corven *et al.,* 1989; Moolenaar, 1995), but has no effect on the proliferation of Jurkat cells (Tigyi *et al.,* 1994). Hence, the role of PLD in signal transduction may vary between cell types depending on the expression of proteins that generate, metabolize and/or respond to PA and LPA.

In conclusion, these studies establish that expression of phorbol ester-sensitive PLD activity varies between different T-lymphocyte cell lines. Activation of PLD is not required for the activations of either ERK or JNK seen in response to phorbol ester.

Other roles for PLD in the regulation of T-cell function remain open for investigation.

CHAPTER 3

Efffects of Sphingomyelinase Reaction Products on Mitogen-Activated Protein Kinase Activity in T-Lymphocyte Cell Lines

3-1. Introduction

Sphingomyelinase (SMase) can be activated in response to extracellular signals, such as TNF-α,1α,25-dihydroxyvitamin D_3, and complement (Hannun, 1994). The SMase that is activated by extracellular signals in mammalian cells appears to be a neutral, Mg-dependent enzyme that is localized to the plasma membrane. The hydrolysis of sphingomyelin by SMase produces ceramide, a proposed lipid mediator (Kolesnick, 1991). Ceramide can effect the activity of protein kinases (Liu *et al.,* 1994) and phosphatases (Dobrowsky and Hannun, 1992), interfere with protein-protein interactions (Kanety *et al.,* 1996), and induce apoptosis (Obeid *et al.,* 1993). For example, in quiescent Swiss 3T3 fibroblasts, ceramide has been shown to stimulate [^{3}H]thymidine incorporation and to act syngergistically with a wide variety of growth factors known to induce proliferation in these cells (Olivera *et al.,* 1992). In contrast, the ability of ceramide to induce apoptosis has been demonstrated in both hemopoietic and nonhemopoietic cell lines, including fibroblasts and fibrosarcoma cell lines (Hannun and Obeid, 1995). Ceramide is considered by many researchers to be an endogenous mediator of apoptosis that may modulate signal

transduction through AP-1 activation (Sawai *et al.,* 1995; Hannun and Obeid, 1995). Ceramide has also been shown to inhibit phorbol-ester-induced PLD activation as well as interfere with PKC-mediated activation of PLD (Venable *et al.,* 1996). The mechanism of activation of neutral SMase has not been established, but may involve activation of proteases (Pronk *et al.,* 1996).

Consistent with its proposed role in programmed cell death, ceramide has been shown to activate JNK kinases in some cell types (Kyriakis *et al.,* 1994; Westwick *et al.,* 1995; Welsh, 1996). In addition, ceramide has been reported to activate ERKs in HL-60 cells (Raines *et al.,* 1993). ERKs are activated by growth factors and other agonists via a ras-dependent protein phosphorylation cascade involving Raf-1 and MEK (Seger and Krebs, 1995). JNKs can be activated in response to both cytotoxic agents (Kyriakis *et al.,* 1994; Chen *et al.,* 1996) and growth factors (Minden *et al.,* 1995). JNKs are activated downstream of the small GTP-binding protein rac (Minden *et al.,* 1995; Coso *et al.,* 1995) via a phosphorylation cascade involving PAK (Bagrodia *et al.,* 1995) and SEK (Moriguchi *et al.,* 1995). The tyrosine kinase PYK2 can activate JNK via ras/rac (Tokiwa *et al.,* 1996) (Figure 3-1). The duration of JNK activation

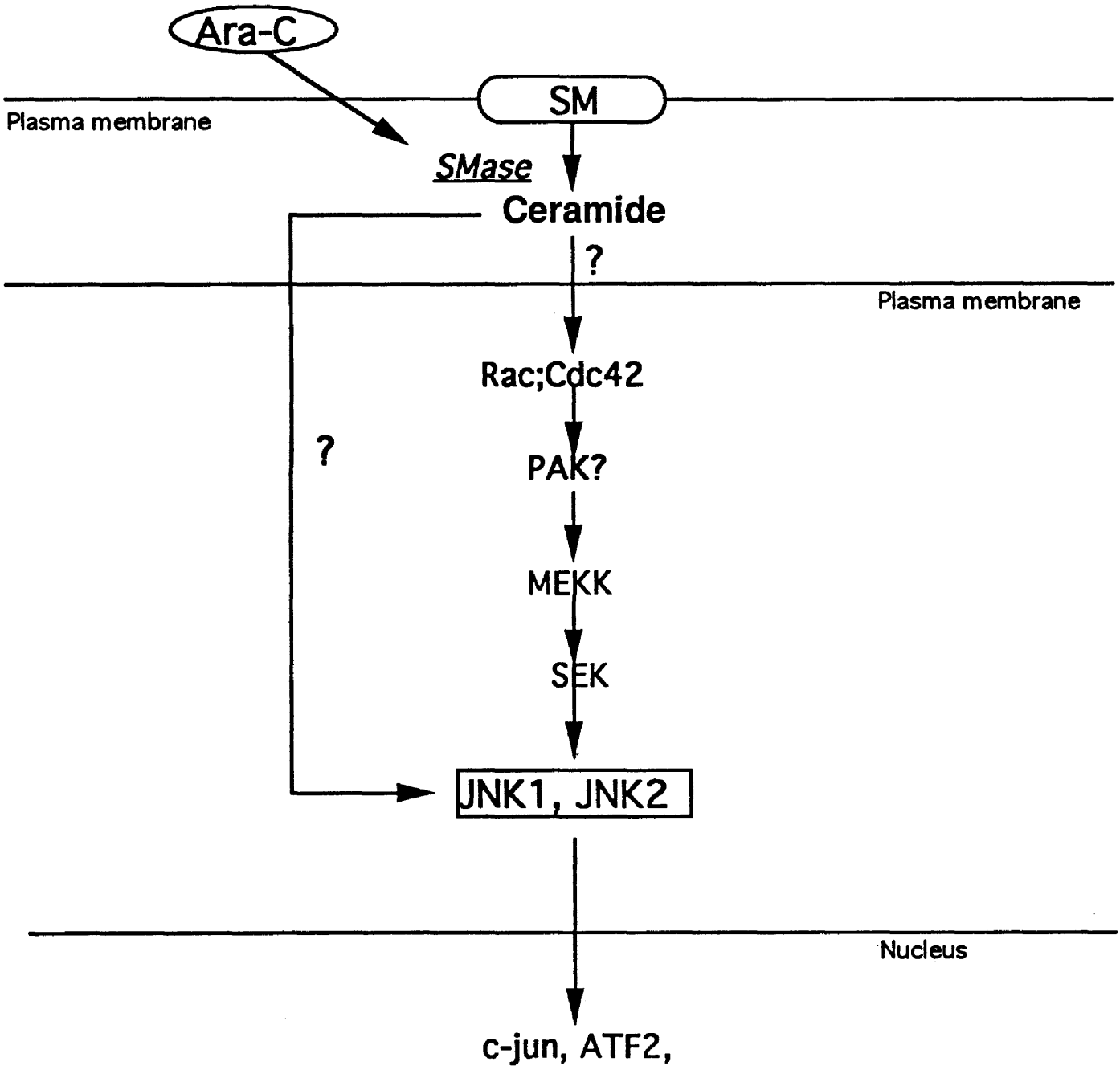

FIGURE 3-1. Proposed mechanism for SMase activation leading to JNK signaling.

The antileukemic drug, Ara-C activates a neutral SMase in the plasma membrane resulting in hydrolysis of sphingomyelin (SM) to ceramide and phosphocholine. It is unknown whether ceramide indirectly activates JNK (e.g., via rac,cdc42), or directly activates kinases upstream of JNKs. JNK activation leads to activation of the transcription factors c-jun and ATF2.

may be important with respect to the signal transduced, since physiologic stimuli typically induce transient activation, while gamma radiation induces persistent activation (Chen *et al.,* 1996).

In T-lymphocytes, JNKs are synergistically activated in response to a combination of phorbol ester and ionophore, or in response to ligation of the T-cell receptor (Su *et al.,* 1994). These stimuli result in expression of interleukin-2 (IL-2) and stimulation of T-cell proliferation. Although JNK activation appears to be most important in the regulation of IL-2 expression, both ERKs and JNKs are activated by T-cell ligation in T-cells (Su *et al.,* 1994). Recently, it was shown that T-cell receptor-mediated activation of both ERKs and JNKs is blocked in anergic T cells (Fields *et al.,* 1996; Li *et al.,* 1996). In non-lymphocytic cells, ERKs and JNKs are proposed to play opposing roles in the regulation of apoptosis, with JNK activation inducing cell death and ERK activation opposing it (Xia *et al.,* 1995). However the existing literature suggests that JNK activation, but not ERK activation, is required for T-cell proliferation.

The studies cited above suggested that ceramide may activate members of the MAPK family. We examined whether this was the

case in T-cells. Specifically, this study addresses whether ceramide is a second messenger inducing JNK or ERK activation in T-cell lines.

Several strategies have been used to elevate ceramide levels in mammalian cells. As discussed earlier, several cytokines can increase ceramide in intact cells (e.g., TNFα, IL-1). However, a response to these agonists is dependent on expression of the appropriate receptor in the cells of interest (Hannun and Obeid, 1995). Long-chain ceramides, similar to those produced endogenously, are not cell-permeable and generally do not mimic the effects of agonists on intact cells. Short-chain ceramides have been widely used to mimic the actions of agonists that increase ceramide levels. C2- and C6-ceramide are cell-permeable, short-chain ceramides shown to elicit responses in a wide variety of cell types. Finally, exogenous bacterial SMase has been reported to elevate ceramide levels in intact cells and to induce signal transduction (Olivera *et al.,* 1992).

Ara-C has been reported to induce acute activation of SMase in HL-60 cells (Strum *et al.,* 1994) and in other cell types (L. Daniels, personal communication). Ceramide can induce apoptosis in HL-60

via induction of AP-1, which contains c-Jun (Sawai *et al.,* 1995). In fibroblasts, Ara-C activates JNKs via the tyrosine kinase c-Abl (Kharbanda *et al.,* 1995). The role of such early signal transduction events in the cytotoxic response to Ara-C have not yet been established.

Ara-C, an anti-leukemic drug (Kharbanda *et al.,* 1994; Strum *et al.,* 1994), is a nucleoside analog that is transported into the cell by a nucleoside transporter and is then sequentially phosphorylated to Ara-CTP:

The cytotoxic metabolite of Ara-C, Ara-CTP, is incorporated into DNA, resulting in slowing of chain elongation and induction of strand breakage (Crisp *et al.,* 1996). Ara-C causes DNA fragmentation and endonucleolytic DNA cleavage, which is a hallmark of apoptosis (Kufe *et al.,* 1984). Ara-CTP also acts as a competitive inhibitor of DNA polymerase. Accumulation of deoxyribonucleoside triphosphates (dNTPs) in response to Ara-C is thought to be responsible for disruption of DNA synthesis in susceptible leukemia cells (Crisp *et al.,* 1996). Since Ara-C appeared to be a general

activator of SMase, and was known to be cytotoxic for in T-lymphocytes, it was used as a model stimulus in our studies.

3-2. Methods

Cell Culture and Incubations

Jurkat cells and wild-type EL4 cells (Gause *et al.,* 1993) were originally provided by Drs. S. Slivka (Tanabe Research Laboratories) and D. Morris (University of Washington), respectively. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (UBI), non-essential amino acids, and penicillin/streptomycin. Cells were incubated with the desired agents (100 nM PMA, {LC Services}, 100 μM Ara-C {Sigma}, 5 μM ceramide {Sigma}, or 10 U/ml bacterial SMase, {Sigma}) in culture medium at 37°C, washed with ice-cold phosphate-buffered saline (PBS), resuspended in lysis buffer (20 mM HEPES {pH 7.5}, 80 mM ß-glycerophosphate, 10 mM EGTA, 2 mM EDTA, 2 mM DTT), sonicated, and sedimented by centrifugation (Beckman) at 100,000 x g for 20 minutes at 4°C. The supernatant (cytosol) and pellet (membranes) were used for ERK and SMase assays, respectively. Extracts prepared in whole-cell lysis buffer (20 mM Tris {pH 7.4}, 137 mM NaCl, 25 mM ß-glycerophosphate, 2 mM sodium

pyrophosphate, 2 mM EDTA, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 mM benzamidine, 0.5 mM DTT) were sedimented by centrifugation at 100,000 x g for 30 minutes at 4°C. The supernatant was used for JNK assays.

SMase Assay

SMase activity was assayed *in vitro* using BODIPY-sphingomyelin (N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosyl-phosphocholine, BSM, Molecular Probes, catalog #D-3522). BODIPY is a trademark of Molecular Probes, Inc. The assay was performed as previously described for phospholipase D (refer to Chapter 2), except that magnesium was included. The reaction mixture (12.5 μl) contained 10 μg membrane protein, 100 μM BSM, 150 mM NaCl, 25 mM HEPES (pH 7.5), 5 mM EGTA, 1 mM EDTA, 40 mM ß-glycerophosphate, 200 μM octylglucoside, and 3 mM $MgCl_2$. The concentration of 3mM $MgCl_2$ was determined to be optimal for measurement of SMase activity in Jurkat membranes (Ella and Meier, unpublished observations). BSM

was solubilized prior to each assay by brief sonication in buffered solution (0.5 mM octylglucoside, 400 mM NaCl, 66 mM MES {pH 6.0}). Reactions were incubated for 60 minutes at 30°C. A 5-µl aliquot was applied to silica gel G60 thin-layer chromatography plates (Merck) and developed in chloroform/methanol/water/ acetic acid (45:45:10:2). Results were imaged and/or quantitated using a FluorImager (Molecular Dynamics) or scanning fluorescence densitometer (Helena Laboratories). Product formation was calculated as a percent of total fluorescence to normalize for loading. Protein concentrations were determined using Coomassie reagent (Pierce).

Protein Kinase Assays

The methods used to assess ERK and JNK activity were described in Chapter 2.

3-3. Results

Characterization of SMase Activity

A fluorescent assay was used to characterize SMase activity in cell membranes. This assay has been used to characterize a yeast

SMase (Ella *et al.,* 1997). The substrate used, BSM, has a BODIPY fluorophore conjugated to the fatty acid at the 2-position of the sphingoid backbone. Similarly substituted sphingolipids can serve as substrates for neutral SMase, but not for lysosomal acidic SMase (Levade *et al.,* 1991). In other words, acid SMases cannot hydrolyze sphingomyelins with a bulky substituent at the 2-position.

As shown in Figure 3-2, membranes from Jurkat and EL4 cells convert BSM to BODIPY-ceramide in the presence of magnesium. No SMase activity was present in cytosol. Similar results were obtained in the absence and presence of dithiothreitol, an inhibitor of acid SMase (Maruyama and Arima, 1989) (data not shown). The pH optimum was 7.5 for Jurkat membranes (data not shown). These data indicate that the *in vitro* assay detects a neutral SMase activity in a T-cell line.

Effects of Ara-C and PMA on SMase Activity

The efects of agonists on SMase activity in T-lymphocytes had not been previously studied. The effects of Ara-C and PMA on membrane SMase activity were therefore examined. Ara-C consistently induced an increase in SMase activity. In Jurkat cells,

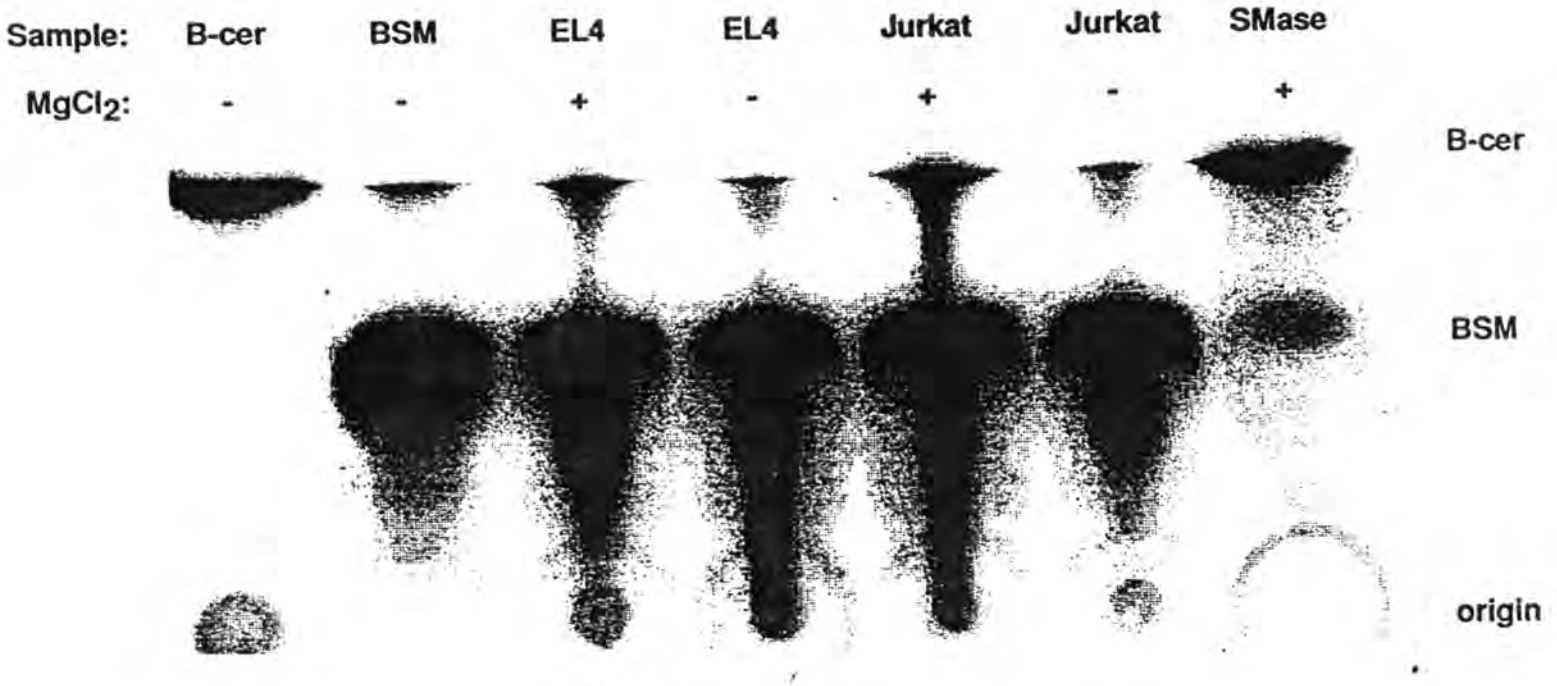

FIGURE 3-2. SMase activity in Jurkat and EL4 cells.

SMase activity was assessed in membranes using an *in vitro* fluorescent assay with BSM as substrate in the absence and presence of 3mM $MgCl_2$. The thin-layer chromatography plate was imaged by a FluorImager (printed with reverse contrast). BSM, BODIPY-ceramide (B-cer), and bacterial SMase incubated with BSM, were chromatographed as standards. $MgCl_2$ did not alter the amount of B-cer present in the absence of membranes or SMase (data not shown).

activation of SMase was detected within 5 minutes, peaked at 10 minutes, and was maintained for at least 60 minutes (Figure 3-3A). SMase activity was not increased when Ara-C was added directly to membrane preparations (data not shown). Ara-C also activated SMase in EL4 cells (Figure 3-3B). The maximal effective dose of Ara-C in Jurkat was 100 μM, as determined at 10 minutes (Figure 3-4). Treatment of Jurkat cells with higher doses of Ara-C (300 μM) did not increase SMase activity (data not shown). Neither PMA (Figure 3-3B) nor ionomycin (with or without PMA) (Figure 3-5), activated SMase in Jurkat or EL4 cells. SMase activity in Ara-C-treated cells was 142.3 ± 3.8% of control in EL4, and 146 ± 11.6% of control in Jurkat cells (mean ± S.D., n = 3 experiments for each cell line). It has been suggested that ceramide only exerts a cytotoxic effect following its intracellular accumulation (Testi, 1996). We therefore attempted to measure ceramide levels in intact Jurkat cells. A published procedure utilizing metabolic labeling of cells with [^{3}H]-palmitic acid was used (Strum *et al.,* 1994). This approach was similar to that used in our laboratory to measure PLD activity. The conditions that were tested included times of incubation with Ara-C from 0 to 30 minutes. The solvent system used to separate

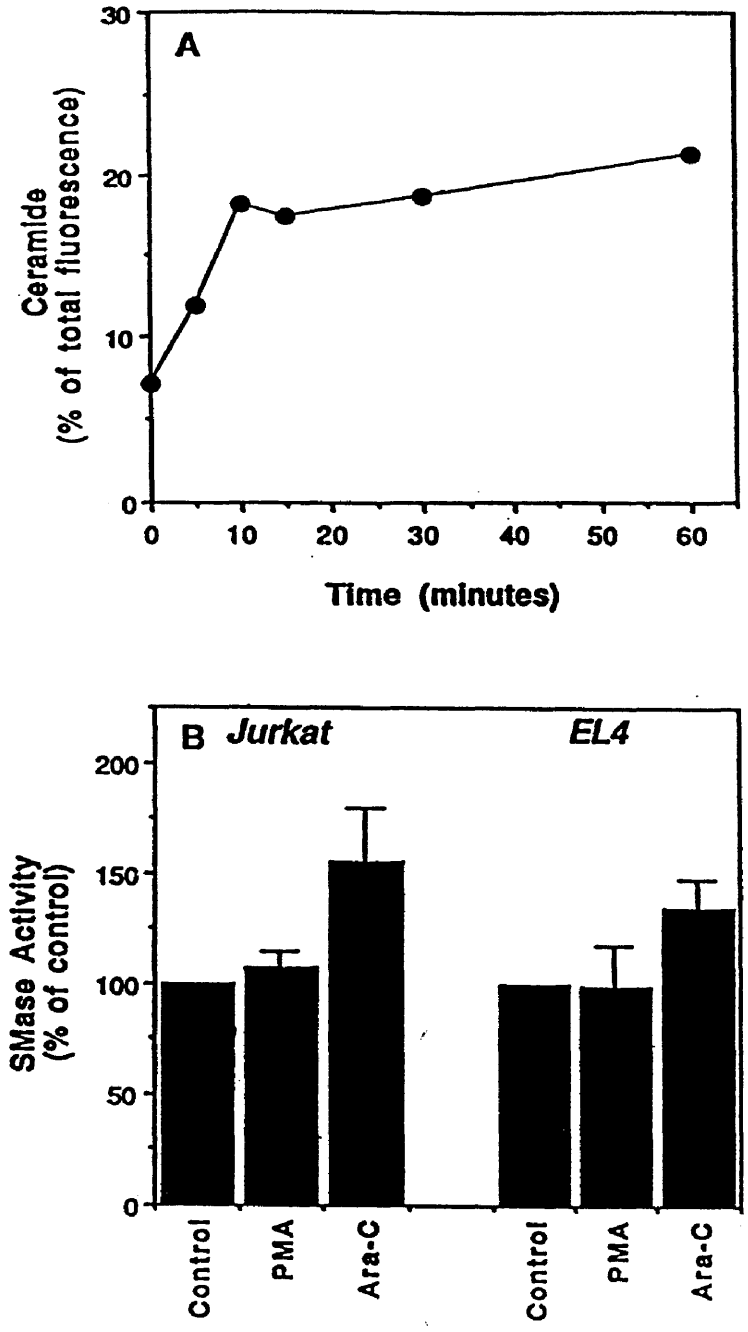

FIGURE 3-3. Effects of Ara-C on SMase activity in Jurkat and EL4 cells.

SMase activity was measured *in vitro*. Panel A: Cells were incubated for the indicated times with 100 μM Ara-C. Ceramide production is expressed as percent of total fluorescence. Panel B: Cells were incubated for 10 minutes with 100 μM Ara-C or 100 nM PMA. Ceramide production, as percent of the value for untreated control cells, is the mean ± S.E.M. from six (Jurkat) or three (EL4) independent experiments.

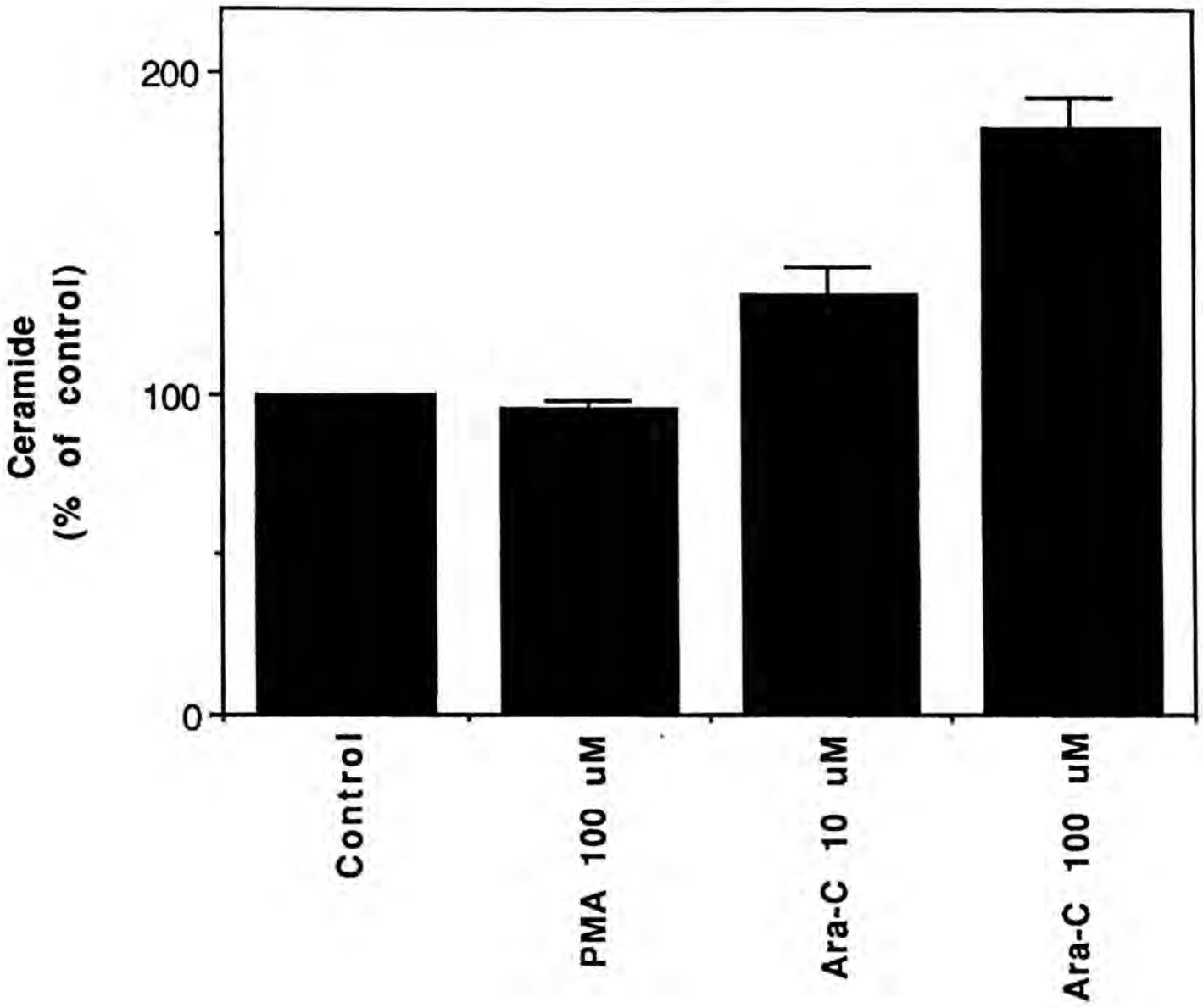

FIGURE 3-4. Effects of Ara-C on SMase activity in Jurkat. Membrane SMase was measured *in vitro* using a fluorescent assay. Single aliquots of Jurkat cells were incubated for 10 minutes with 10 μM Ara-C, 100 μM Ara-C, or 100 nM PMA. Ceramide production is expressed as percent of total fluorescence. Each data point represents the mean ± S.D. of values obtained from duplicate samples of cells.

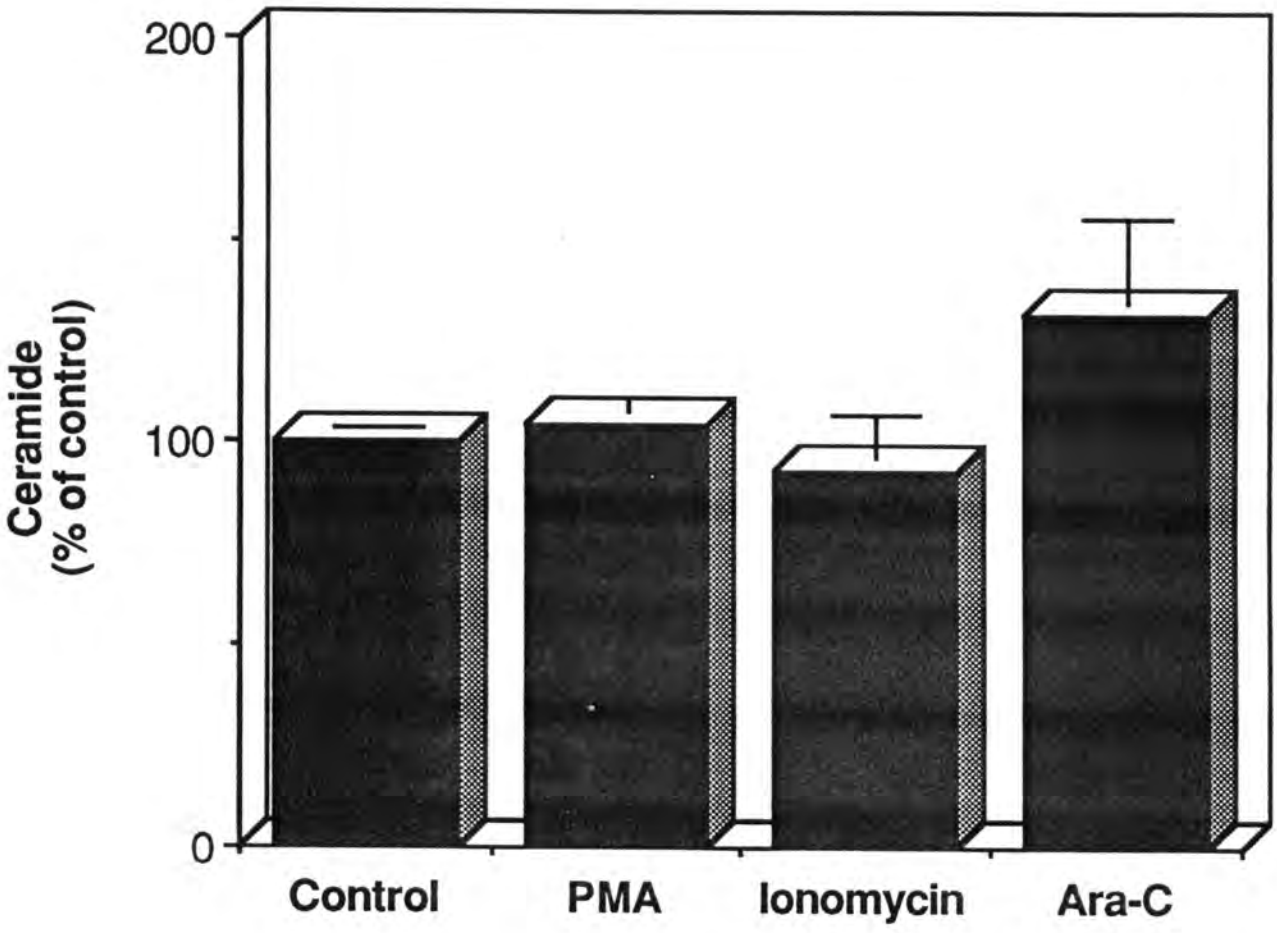

FIGURE 3-5. Effects of PMA and ionomycin on SMase activity in Jurkat.

Membrane SMase activity was measured *in vitro* using a fluorescent assay. Single aliquots of Jurkat cells were incubated for 10 minutes with 100 nM PMA, 2 μM ionomycin, or 100 μM Ara-C. Ceramide production, as percent of the value for untreated control cells, is the mean ± S.E.M. from three independent experiments.

labelled lipids was effective in resolving ceramide from other products (data not shown). Unfortunately, no increase in ceramide levels was detected in these experiments. Since our major goal was to determine whether ceramide acts directly as a second messenger to induce MAPK activation, measurement of ceramide accumulation was not further pursued. The following sections describe more pharmacologic approaches to address the role of ceramide in T-cells.

Effects of Ara-C and PMA on ERK and JNK Activities

The effects of Ara-C, PMA, and other agents on ERK activity were examined. PMA activated ERKs in Jurkat in 10 minutes, while Ara-C had no effect (Figure 3-6A). C2-ceramide, a cell-permeable derivative (Gomez-Munoz *et al.,* 1994), did not affect ERK activity. Similar results were obtained with EL4 (Figure 3-6B), in which PMA activates ERKs (Chapter 2).

JNKs were synergistically activated in Jurkat (Figure 3-7A) and EL4 cells at 10 minutes by a combination of PMA and ionomycin, as previously reported (Su *et al.,* 1994; Bradshaw *et al.,* 1996). PMA alone slightly activated JNKs, while ionomycin, Ara-C, C2-ceramide (5 and 50 μM), and crude ceramide neither activated JNKs at 10

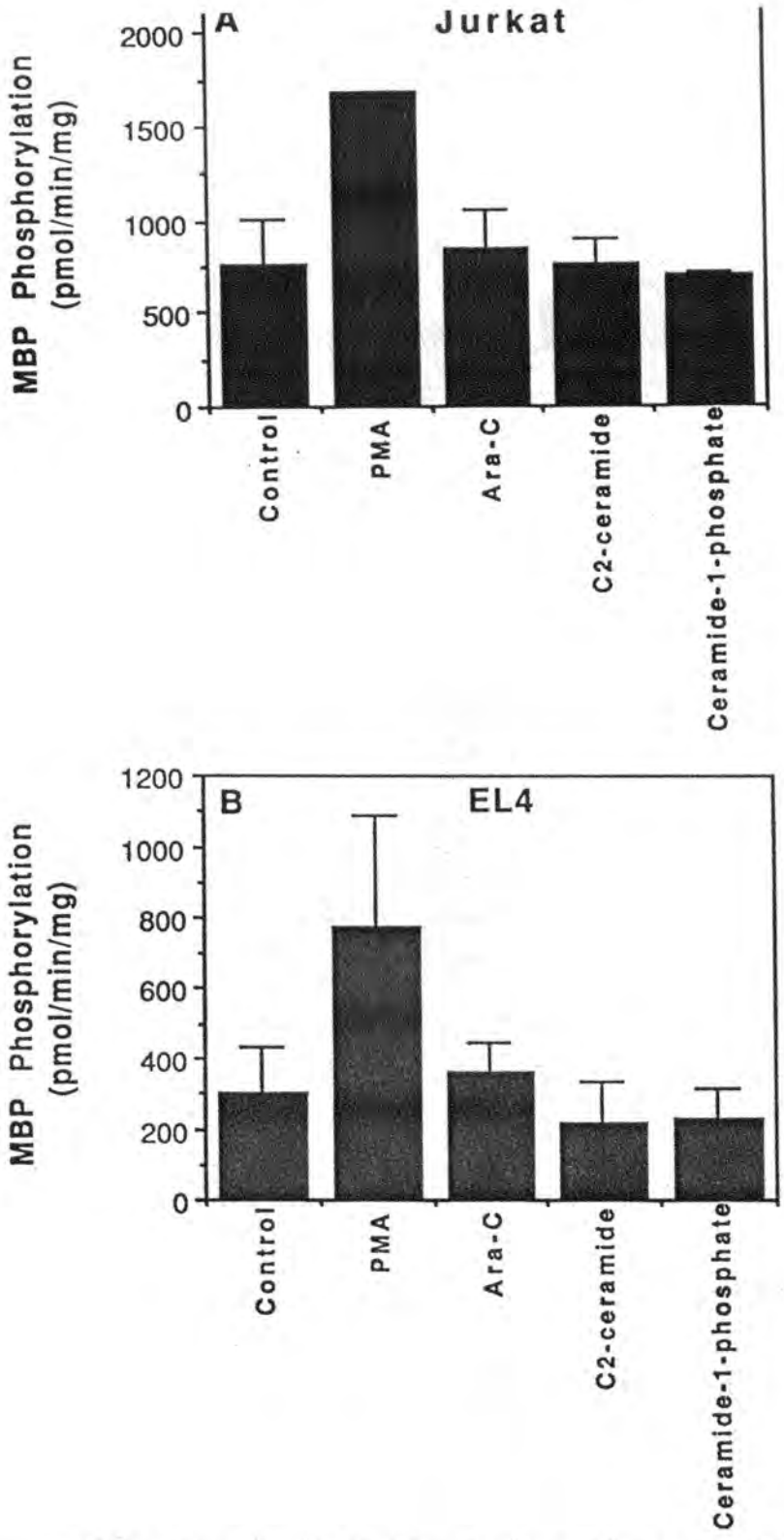

FIGURE 3-6. Effects of Ara-C and C2-ceramide on ERK activity in Jurkat and EL4 cells.

In the same experiment, both cell types were incubated for 10 minutes with 100 nM PMA, 100 μM Ara-C, or 5 μM C2-ceramide. Results from Jurkat cells are shown in panel A, and from EL4 cells in panel B. Cytosolic ERK activity is the mean ± S.D. of values obtained from duplicate aliquots of cells.

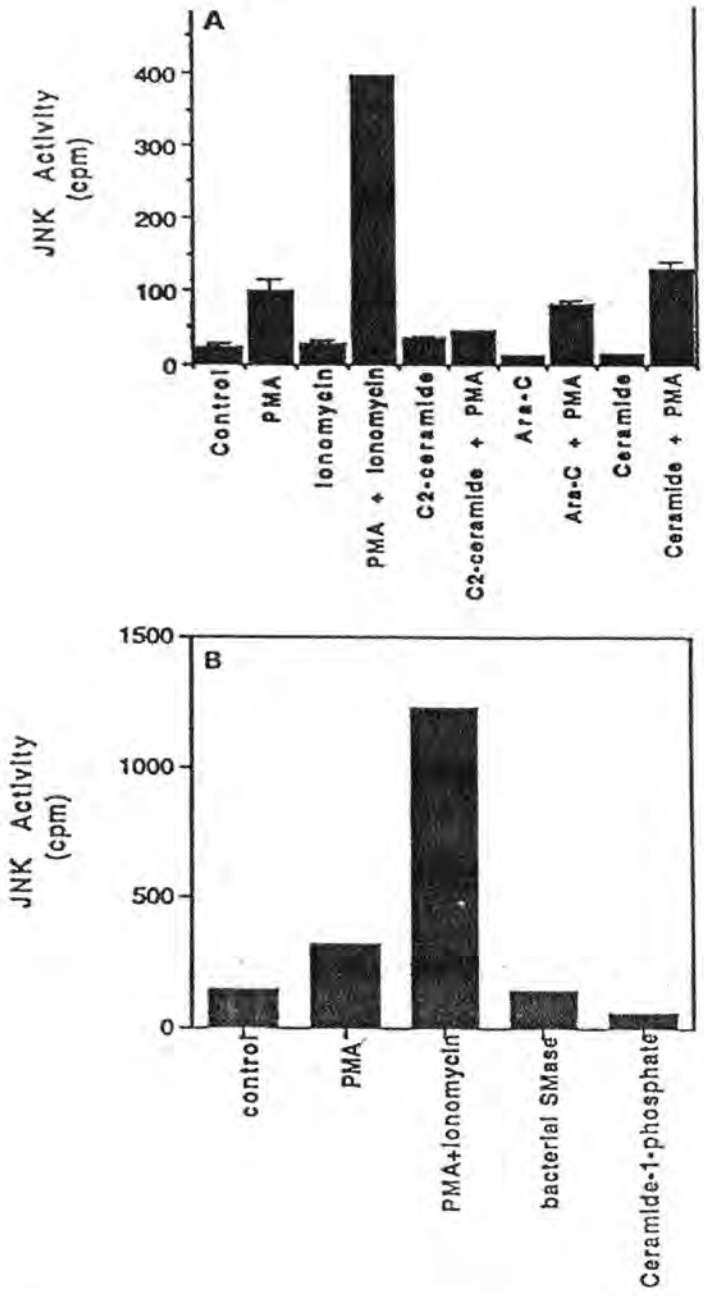

FIGURE 3-7. Effects of Ara-C and ceramides on JNK activity in Jurkat cells.

In panel A, Jurkat cells were incubated for 10 minutes with 100 nM PMA, 2 μM ionomycin, PMA + ionomycin, 100 μM Ara-C, Ara-C + PMA, 5 μM C2-ceramide, C2-ceramide + PMA, 5 μM ceramide, or ceramide + PMA. JNK activity in whole cell extracts is expressed as the mean ± S.D. from duplicate aliquots of cells. In panel B, single aliquots of Jurkat cells were incubated for 10 minutes with 100 nM PMA, PMA + Ionomycin, 10 U/ml bacterial SMase, or 5 μM ceramide-1-phosphate. Similiar results were obtained in two separate experiments.

minutes (Figure 3-7A) nor affected the response to PMA (Figure 3-7A). Ceramide-1-phosphate (5 μM) and SMase from *S. aureus* (10 U/ml) likewise did not activate JNK at 10 minutes (Figure 3-7B). Higher doses of ceramides did not affect JNK activity (data not shown).

Since cytotoxic agents can cause a slowly-induced and persistent stimulation of JNKs (Park *et al.,* 1996), the effects of more prolonged incubations with Ara-C were examined. As shown in Figure 3-8A, JNK activation was observed when Jurkat cells were incubated for longer times with Ara-C. JNK activation was apparent by 90 minutes, and persisted for at least 120 minutes. Similar results were obtained in EL4, in which JNK activation was observed only after 120 minutes (Figure 3-8B).

Ara-C did not activate ERKs at 10-120 minutes in Jurkat (Figure 3-9) or EL4 (data not shown).

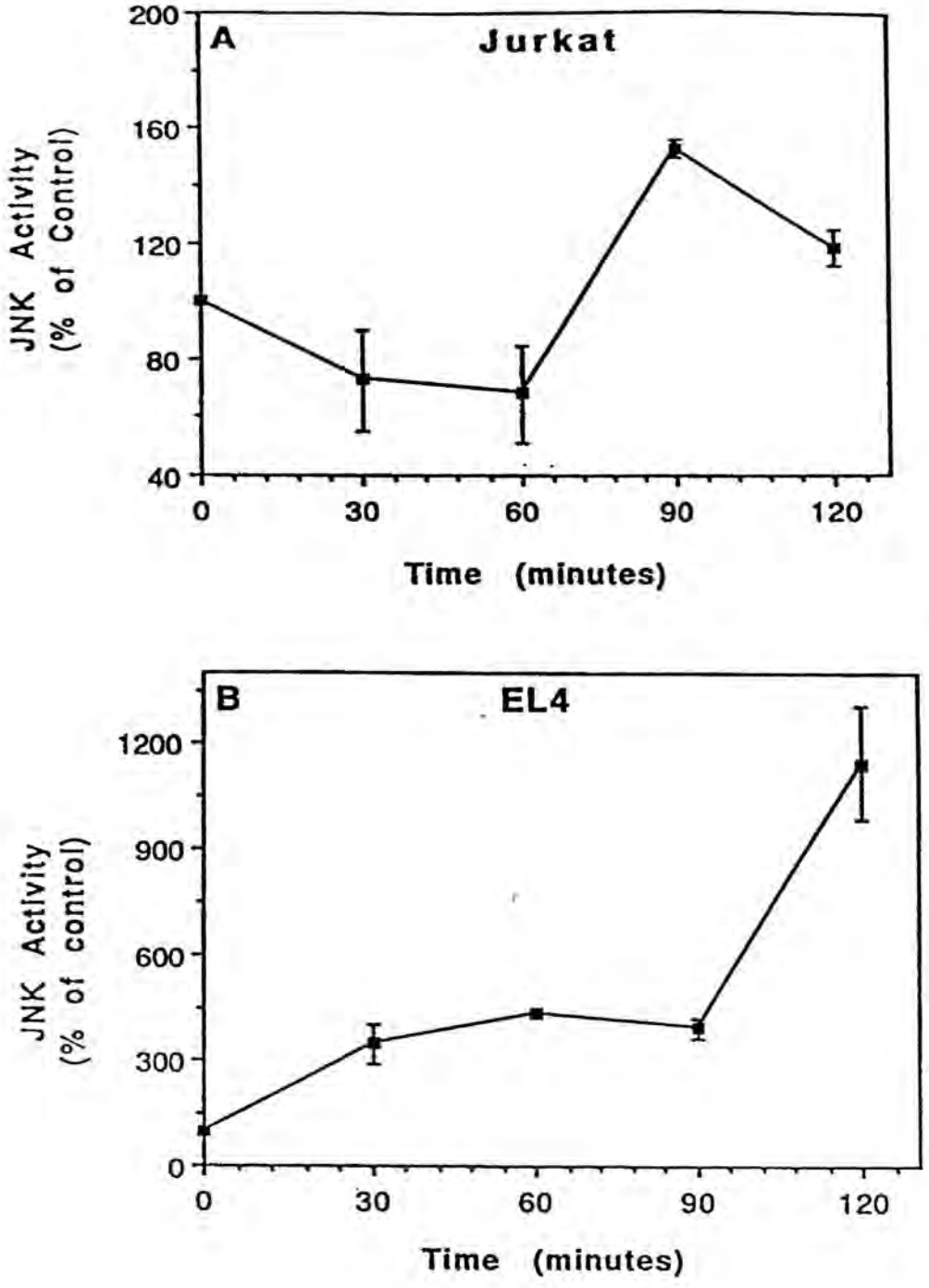

FIGURE 3-8. Effects of prolonged treatment with Ara-C on JNK activity in Jurkat and EL4 cells.

Jurkat (panel A) and EL4 (panel B) cells were incubated for the indicated times with 100 μM Ara-C. JNK activity is the mean ± S.D of control (untreated cells) obtained from two independent experiments using duplicate samples of cells.

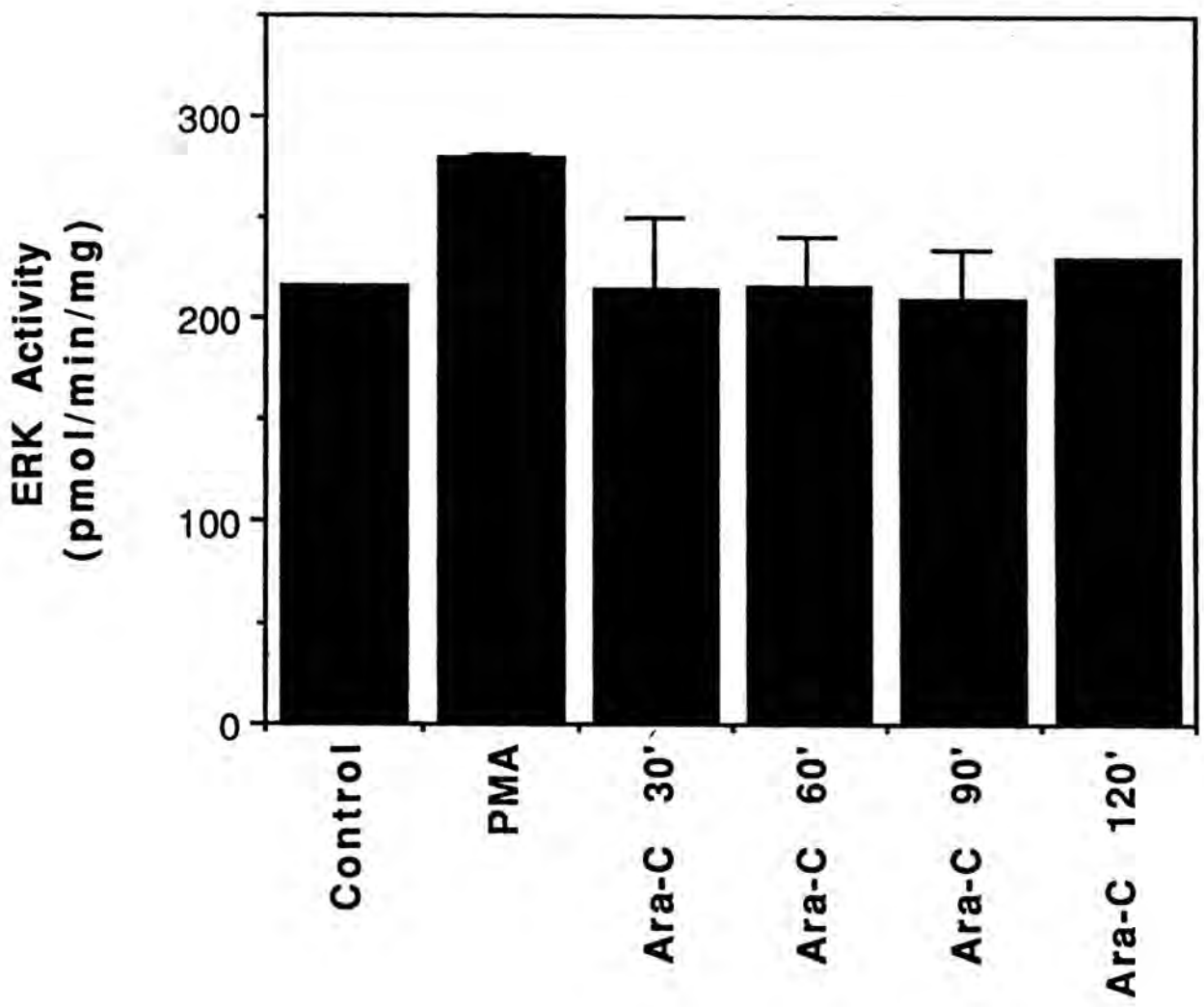

FIGURE 3-9. Effects of prolonged treatment with Ara-C on ERK activity in Jurkat cells.

Jurkat cells were incubated with 100 μM Ara-C for the indicated times or with 100 nM PMA for 10 minutes. Cytosolic ERK activity, measured as MBP kinase activity, is the mean ± S.D of values obtained from duplicate aliquots of cells.

3-4. Discussion

We have examined the potential role of SMase activation in regulating the activities of the ERK and JNK kinases in two T-cell lines. We show for the first time that Jurkat and EL4 cells express a membrane-localized neutral SMase. We also show that SMase activity is increased in membranes prepared from Ara-C-treated cells. One previous report indicated that the activated state of neutral SMase could be preserved in a broken-cell preparation (Strum *et al.,* 1994). With the exception of divalent cations, we and others have not identified factors that activate neutral SMase in membrane preparations. A role for proteases in SMase activation has been proposed on the basis of intact cell studies (Wright *et al.,* 1996). Until suitable molecular probes are developed for neutral SMase, it will not be possible to determine whether the enzyme is activated by covalent modification (e.g., phosphorylation), translocation, proteolysis, or via protein-protein interactions.

Ceramide does not appear to activate the ERK cascade in Jurkat or EL4 cells. These results contrast with those observed in one study of HL-60 cells, in which ceramide induced ERK activation (Raines *et al.,* 1993). However, in another study, ceramide inhibited

ERK activity in HL-60 cells (Westwick *et al.,* 1995). Since ERKs can be activated by PKC activators in both Jurkat and EL4 cells (Chapter 2), the lack of effect of Ara-C suggests that Ara-C does not significantly stimulate protein kinase C-mediated pathways in these T-cell lines. We were unable to detect any significant inhibitory effect of Ara-C on PMA-induced ERK or JNK activation in this study.

JNKs were not activated at early time points after Ara-C addition, when SMase was activated, but were activated in response to more prolonged treatment with Ara-C. This time course does not correlate with the acute (10-minuté) activation of SMase by Ara-C in this and a previous study (Strum *et al.,* 1994). Since SMase activity remains elevated for at least one hour, it is possible that accumulation of ceramide (or its metabolites) is a direct signal for JNK activation. However, we did not detect JNK activation following a 10-minute incubation with cell-permeable ceramides. Bacterial SMase induced acute JNK activation in hepatoma cells (Kyriakis *et al.,* 1994) and HL-60 cells (Westwick *et al.,* 1995). Interpretation of these responses may be complicated by the fact that bacterial SMase preparations can contain additional phospholipase activities (Ella and Meier, unpublished observations). Ceramide induces c-Jun

within one hour in HL-60 cells (Sawai *et al.,* 1995), an effect that could be consistent with a delayed time course of JNK activation. Taken together, these results suggest that ceramide can induce long-term effects on mammalian cells. Such effects, though of potential physiologic importance, make it more difficult to determine whether ceramide is truly a "second messenger."

The role of SMase activation in the induction of apoptosis by Ara-C in cells of hematopoietic origin remains unclear. Although JNKs can be activated by "stressful" cytotoxic stimuli, their positive involvement in T-cell proliferation (Su *et al.,* 1994) suggests that their roles can vary between cell types. The balance of JNK and ERK activities can determine the final cellular response (Xia *et al.,* 1995). In T-cell activation, both ERKs and JNKs are activated. In contrast, Ara-C activates only JNKs in Jurkat and EL4 cells (this study) and in HL-60 cells (Westwick *et al.,* 1995). The kinetics of the response may also be important. Lethal irradiation induces a prolonged activation of JNK that is not detected until after one hour (Su *et al.,* 1994). In fibroblasts, Ara-C activates JNKs only after two hours (Kharbanda *et al.,* 1995). If activation of JNKs leads to apoptosis in the absence of ERK activation, perhaps it is

necessary that this response be initiated slowly (i.e., only in response to a persistent cytotoxic insult). Finally, apoptosis can be induced by withdrawal of positive signals for cell proliferation (Ishizaki *et al.,* 1995). Both ERKs and JNKs are activated by co-stimulation of T-cells. Thus, activation of JNK in the absence of ERK activation may be an important aspect of the action of cytotoxic agents that activate SMase in T-cells.

CHAPTER 4

Expression and Regulation of Phospholipase D Activity in Mammalian Cell Lines

4-1. Introduction

Phospholipase D (PLD), which catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA), is proposed to play an important role in signal transduction for many types of cells. PLD is activated in mammalian cells in response to growth factors, agonists that activate phosphatidylinositol-specific PLCs (PIP_2-PLCs), and phorbol esters (Exton, 1990). It is not clear how many forms of PLD exist. Although cytosolic forms have been described, the regulated forms appear to be membrane-bound. The mechanism of activation of PLD may vary between isoforms. Several factors have been implicated in the regulation of PLD activity, including calcium, PKCs, protein-tyrosine kinases, and GTP-binding proteins (Exton, 1994). The GTP-binding proteins ARF and Rho activate some forms of PLD (Exton *et al.,* 1992; Cockcroft, 1992; Bowman *et al.,* 1993; Liscovitch *et al.,* 1994; Siddiqui *et al.,* 1995; Brown *et al.,* 1995). PIP_2 acts as a cofactor for various forms of PLD, including ARF-dependent forms. PLDs may be synergistically regulated by PKC, ARF, and Rho (Ohguchi *et al.,* 1996; Singer *et al.,* 1996).

PLD can be activated by PKC via mechanisms that may or may not involve protein phosphorylation. Olson and co-workers (1991)

have reported that PMA-induced PLD activation is dependent on ATP in the neutrophil cell-free system. Additional studies have shown that the effect of ATP on PKC-mediated PLD activation is mediated by phosphorylation in human neutrophils (Lopez *et al.*, 1995). Membrane-bound PLD can be activated by PKC in a phosphorylation-independent mechanism in Chinese hamster lung fibroblasts (Conricode *et al.*, 1992). In these cells, the activation of PLD by PKC was observed in the absence of ATP, suggesting that PKC may activate PLD by an allosteric mechanism without ATP-dependent phosphorylation (Conricode *et al.*, 1992). In liver membranes, stimulation of PLD by PKC likewise does not require ATP (Lopez *et al.*, 1995). In HL-60 cells, Ohguchi and co-workers (1995) have shown that PKC-mediated PLD activation is suppressed by R031-8425, a potent PKC inhibitor. These studies imply that a novel, phosphorylation-independent mechanism exists for PLD activation by PKC (Morris *et al.*, 1996).

Recently, specific PKC isozymes have been implicated in the regulation of PLD. In HL-60 cells, PLD activity was found in partially-purified PKC fractions containing α, βI, βII, and γ isozymes (Ohguchi *et al.*, 1996). PKCα and -β, but not -γ, were able to activate

membrane-bound PLD (Ohguchi *et al.,* 1996). Among these isozymes, PKCα was most effective in activating PLD. One report showed that Rho was required for activation of PLD by G-protein-coupled receptors, but not by PKC (Hess *et al.,* 1997). However, other investigators have found that PKC acts additively or synergistically with Rho (Singer *et al.,* 1996). Thus, the mechanism by which PKC activates PLD remains to be established.

Our laboratory has raised an antibody against a 12-amino acid portion of the yeast PLD sequence that is conserved between plants and yeast. Ten amino acids (83%) of this sequence are conserved in human PLD1a and 1b, (Hammond *et al.,* 1997). This antibody recognizes yeast and plant PLDs, as well as a 120-kDa band in a variety of mammalian cell lines. The 120-kDa band is not observed in EL4 cells, which lack significant PLD activity, but is observed in Jurkat cells, which express PLD activity (refer to Chapter 2). The 120 kDa band is present mainly in membranes, and does not translocate from cytosol to membranes upon treatment of Jurkat cells with PMA. A 50 kDa band has also been observed in both cytosol and membranes from a variety of cell lines. This band may be an additional isoform of PLD. Additional bands of approximately

90- and 95- kDa are seen in some cells.

hPLD1 can be activated *in vitro* by ARF, PIP_2, and PKCα (Morris *et al.,* 1996). Whether this is the major form of PLD regulated by agonists is not clear. The mechanism by which PKCα regulates the activity of hPLD1 has not been established. The subcellular localization of hPLD1 has not been reported. Moreover, as discussed in Chapter 1, there may be more than one form of agonist-activated PLD. This chapter describes studies designed to address some of these issues.

In our *in vitro* assays, agonist-stimulated PLD activity is detected in the absence of added GTP-binding proteins, guanine nucleotides, or ATP. There are at least two possible explanations for the observed retention of agonist stimulation. First, membrane PLD activity may be increased due to translocation of inactive PLD to the membrane, where it may be activated by binding to membrane constituents. Second, PLD already localized to the membrane may be activated by protein-protein (e.g., PLD-PKC) or protein-lipid (e.g., PLD-PIP_2) interactions that are preserved in the membrane preparation used for the *in vitro* assay.

In this study, we explore the use of the anti-PLD antibody to

examine localization and regulation of agonist-activated PLDs in Jurkat T-cells and in PC-3 and LNCaP human prostate cancer cells. The potential for regulation of PLD by phosphorylation was examined. The potential interaction of PLDs with PKCα was also explored.

4-2. Materials and Methods

Cell Culture

Jurkat cells were maintained as described in Chapter 2. PC-3 and LNCaP cells, originally obtained from the American Type Culture Collection, were maintained in F12K medium and RPMI medium, respectively, supplemented with 10% fetal calf serum (Atlanta Biologicals). Cells were incubated with 100 nM PMA (LC Laboratories) in culture medium at 37°C, washed with ice-cold PBS, resuspended in lysis buffer (20 mM HEPES, 80 mM ß-glycerophosphate, 10 mM EGTA, 2 mM EDTA, 2 mM DTT, {pH 7.5}), sonicated, and sedimented by centrifugation at 100,000 x g for 30 minutes at 4°C. The supernatant (cytosol) and pellet (membranes) were used for PLD assays.

Phospholipase D Assays

For PLD assays in intact cells, cells were incubated overnight in complete culture medium (1 ml) containing 10 μCi [^{3}H]-palmitic acid (Dupont NEN). Washed cells were incubated with and without 100 nM PMA (LC Laboratories) in the presence and absence of 0.5% ethanol. Triplicate or quadruplicate samples were used for each experimental condition. Cellular lipids were extracted from the cells with chloroform/methanol and separated by thin layer chromatography as described in Chapter 2. Spots containing phosphatidylethanol (PEt) and phosphatidic acid (PA) were collected for liquid scintillation spectrometry. Results are presented for each product as a percent of the total radioactivity recovered.

For PLD assays in broken cell preparations, cells were incubated in the absence and presence of 100 nM PMA. Cytosolic and membrane extracts were prepared as described in Chapter 2. Membrane PLD activity was assessed *in vitro* using a fluorescent substrate, BPC (Molecular Probes). Each reaction utilized 10 μg of membrane protein. The products were separated on silica gel G60 TLC plates (Merck) and imaged using a FluorImager (Molecular Dynamics). Results were quantitated using a fluorescent scanning

densitometer (Helena Laboratories). Data are quantitated as a percent of the total fluorescent signal recovered from each sample.

Immunoblotting

For the immunoblotting experiments, whole cell extracts were prepared. Cells were incubated with and without 100 nM PMA and incubated in lysis buffer (20 mM HEPES {pH 7.4}, 1% Triton X-100, 50 mM NaCl, 1 mM EGTA, 5 mM ß-glycerophosphate, 30 mM sodium pyrophosphate, 100 µg sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. The lysates were sedimented by centrifugation at 10,000 x g for 10 minutes at 4°C to remove debris. Supernatant proteins were separated by SDS-PAGE on 10 or 12.5% Laemmli gels and transferred to nitrocellulose membranes (Micron Separations, Inc). Primary antibodies to PLD (polyclonal) were prepared by immunizing a rabbit with the peptide CIIGSANINERS linked to keyhole limpet hemocyanin. The peptide represents amino acids 1107-1118 of yeast PLD1 (Ella *et al.,* 1996), corresponding (with two substitutions) to residues 907-918 of human PLD1 (Hammond *et al.,* 1995). Anti-PKCα (monoclonal) was obtained from Transduction

Laboratories. After incubation with primary antibodies, immunoblots were developed using gold-labelled secondary antibodies with silver enhancement (Amersham).

Immunoprecipitation

Whole cell extracts were prepared as described above for immunoblotting. Antibodies to PKCα were incubated with the extracts for 2.5 hours at 4°C with mixing. Protein A-agarose (Santa Cruz Biotechnology) was then added; the incubation was continued for 1 hour. The agarose beads were collected by centrifigation, washed several times with buffer, and then boiled in Laemmli sample buffer. For denaturing conditions, anti-PKCα immunoprecipitations were carried out as described above, except that cells were extracted with boiling solution (1% SDS/10 mM Tris HCl pH 7.4) and then sonicated. Proteins in the immunoprecipitate were subjected to immunoblotting as described above.

4-3. Results

PLD in Jurkat cells

In chapter 2, we showed that PLD is activated in response to PMA in Jurkat cells (Chapter 2). We examined the expression of PLD, using a newly-developed antibody against PLD. We were able to detect a 120-kDa immunoreactive protein in Jurkat cells (Chapter 2). This band, which may correspond to human PLD1 and/or its splice variant (Hammond *et al.*, 1997), was predominantly localized to the membrane fraction. We were also able to detect 90-95-kDa and 50-KDa bands in the cytosol and membrane fractions of Jurkat (data not shown). None of the bands were translocated or showed mobility shifts in response to treatment of cells with PMA.

Additional experiments were performed to determine whether activation of PLD occurs as a result of protein phosphorylation. Membranes from control and PMA-treated cells were incubated in the absence and presence of alkaline phosphatase. As shown in Figure 4-1, PMA-induced PLD activity was substantially decreased in the presence of alkaline phosphatase. Similiar results were also seen for Ara-C-induced SMase activity (data not shown). As a positive control for phosphatase activity under the conditions used,

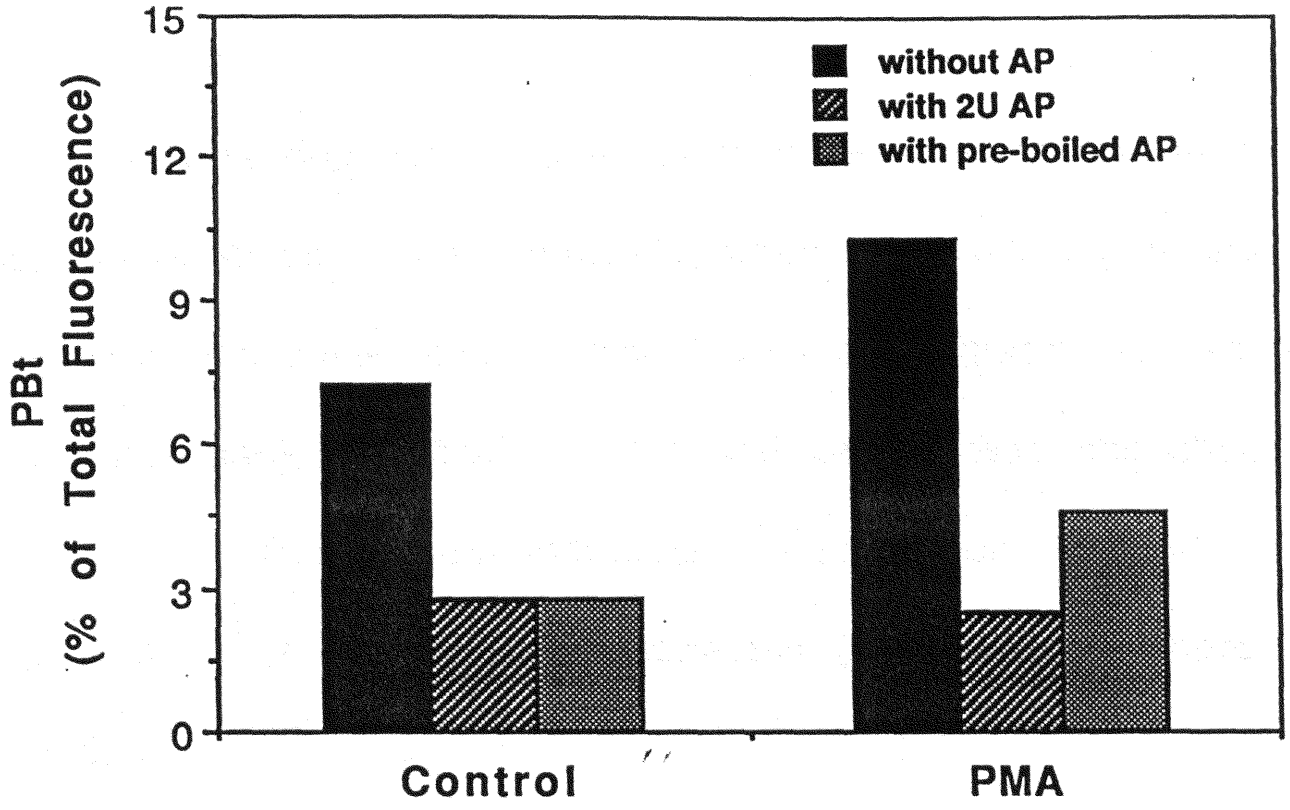

FIGURE 4-1. Effects of alkaline phosphatase on PLD activity.

Jurkat cells were incubated for 10 minutes in the absence and presence of 100 nM PMA. Membrane extracts were incubated with or without 2 U of alkaline phosphatase/12.5 µl reaction buffer at room temperature for 30 minutes, followed by incubation in the presence of BPC and 1% butanol for 1 hour at 30°C. Reaction products were separated using thin layer chromatography. PLD activity was assessed using a FluorImager. Production of BODIPY-phosphatidylbutanol is calculated as a percent of the total fluorescence in each lane. Similiar results were obtained in two separate experiments.

immunoblots were performed using anti-ERK antibody (data not shown). Reversal of the PMA-induced ERK mobility shift indicated that ERKs were dephosphorylated by alkaline phosphatase treatment under the conditions used. However, when the alkaline phosphatase preparation was boiled prior to addition to cell extracts (to denature the phosphatase), inhibition of PLD and SMase was still observed (Figure 4-1). This result suggests a non-specific inhibition by components of the alkaline phosphatase preparation. Therefore, these data do not establish whether phosphorylation is involved in the activation of PLD and/or SMase in Jurkat cells. In view of the difficulties in interpreting the results of phosphatase treatment in crude membrane preparation, we proceeded to determine whether our anti-PLD antibody could be used to study regulation of PLD activity more directly.

PLD in PC-3 and LNCaP Cells

Jurkat cells express relatively low levels of PLD activity, making analysis of PLD regulation more difficult. Our laboratory has characterized several other cellular model systems in which PMA activates PLD activity (Jones *et al.,* 1994; Knoepp et al., 1996; Ella

et al., 1997). For further studies of PLD regulation, we chose to examine the effects of PMA on PLD activity in PC-3 and LNCaP cells (Qi et al., submitted). These cells express high levels of PLD activity, as shown by the *in vitro* assay utilizing BPC as the fluorescent substrate in the presence of 1% butanol (Figure 4-2). PLD activity was present in both PC-3 and LNCaP membranes, as detected by production of phosphatidylbutanol (Figure 4-2). PMA induced increases in PLD activity in both cell lines, as detected using intact cell assays (Qi *et al.*, submitted). Activation of PLD by PMA could also be detected in membrane preparations (Qi *et al.*, submitted), as was the case for Jurkat cells (Chapter 2).

The study was continued by using the anti-PLD antibody to identify putative PLDs. Bands migrating at 120, 95 and 50 KDa were consistently detected in both PC-3 (Figure 4-3) and LNCaP membranes (data not shown). The 120-KDa as well the 95-KDa band were found mainly in membrane fractions. The 95-KDa band could also be detected in cytosolic fractions within these cells. Neither the 120-KDa band nor the 95-KDa translocated from the cytosol to membrane upon PMA treatment. A 50-KDa band is present in cytosol and membranes from a variety of cell lines (Ella, Qi, and Meier,

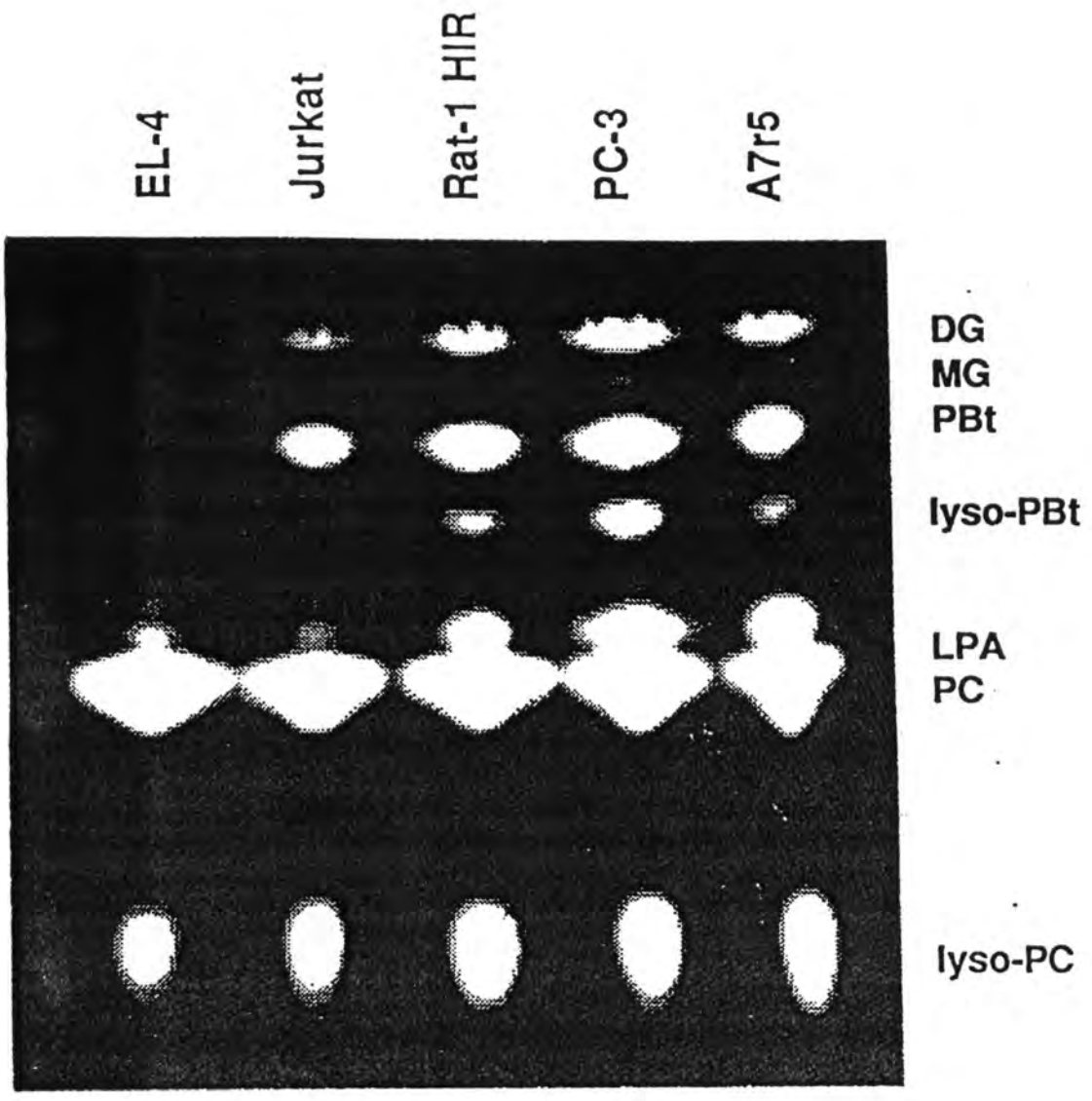

FIGURE 4-2. PLD activity in mammalian cell membranes. Membranes prepared from EL4, Jurkat, Rat-1 HIR, PC-3 and A7r5 cells were incubated for 1 hour at 30°C with BPC in the presence of 1% butanol. Reaction products were separated by thin layer chromatography and imaged using a FluorImager (printed with reverse contrast).

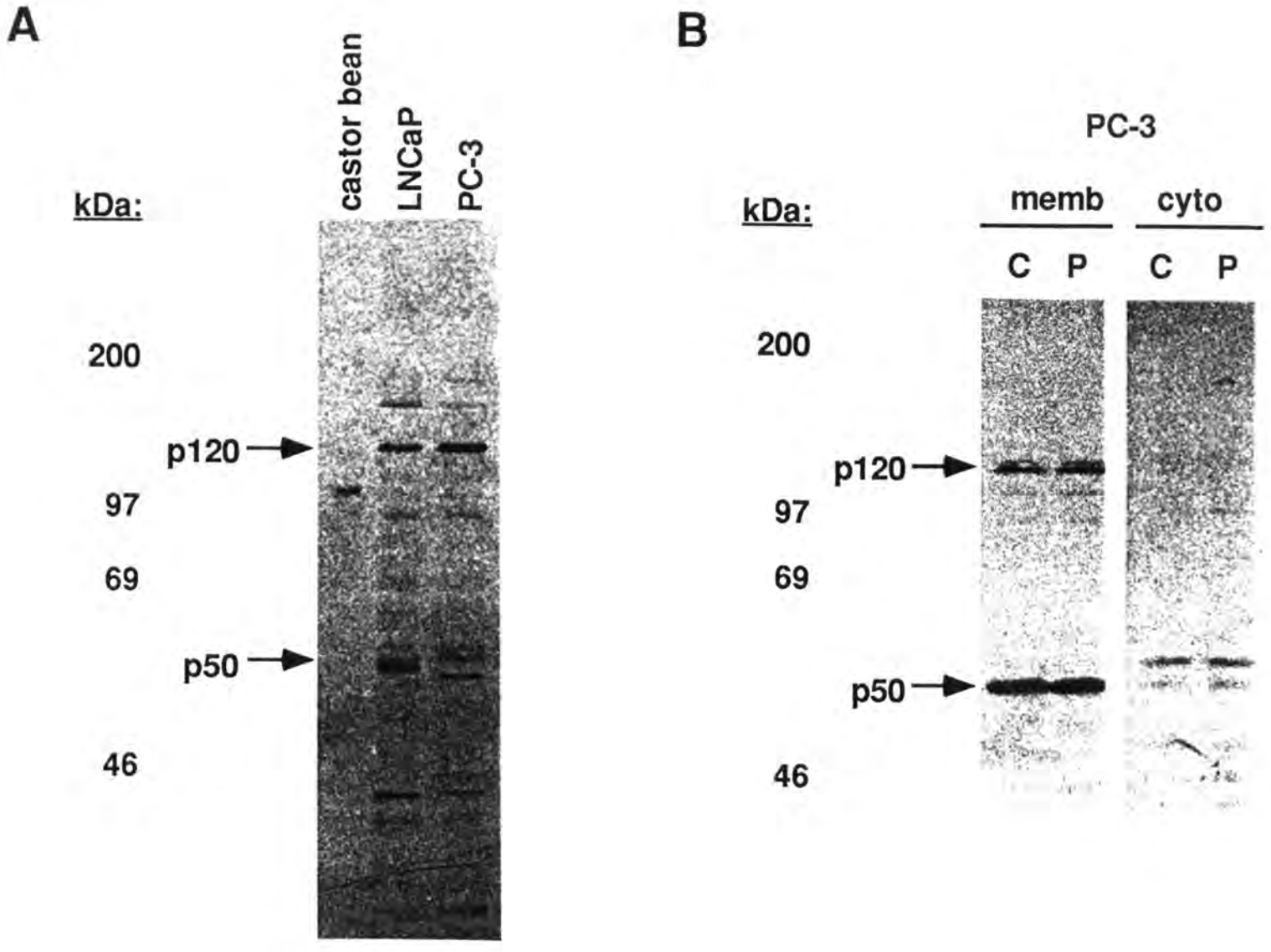

FIGURE 4-3. Immunoblot for PLD in LNCaP and PC-3 cells. In panel A, membranes from LNCaP and PC-3 cells were separated on SDS-PAGE and subjected to immunoblotting for PLD using anti-PLD antibody. Castor bean PLD was used as a positive control. Equal amounts of membrane protein were loaded in each lane. The position of major immunoreactive PLD bands are indicated by arrows. In panel B, PC-3 cells were incubated in the absence ("C") and presence ("P") of 100 nM PMA for 10 minutes. Cytosol and membrane fractions were prepared and immunoblotted with anti-PLD antibody. The migration positions of molecular size markers are shown on the left.

unpublished observations). This band was also seen in PC-3 and LNCaP (Figure 4-3). The 50-KDa band likewise did not translocate in response to PMA treatment. Some signal transduction proteins show apparent mobility shifts upon phosphorylation. However, none of the bands recognized by the anti-PLD antibody in cytosol or membranes shifted in response to treatment of PC-3 cells with PMA.

In order to study effects of agonists on PLD phosphorylation or protein-protein interactions, an immunoprecipitating antibody is extremely useful. We tested the ability of our antibody to immunoprecipitate PLD by performing immunoprecipitations under standard and denaturing conditions. We tested for the presence of immunoprecipitated protein by immunoblotting with the same antibody. Unfortunately, we found that the anti-PLD antibody was unable to immunoprecipitate PLD (Figure 4-4). Although the sequence recognized by the antibody is near the c-terminus, its high degree of conservation suggests that it may lie within the catalytic site. Thus, the epitope may not be available for recognition by the antibody under non-denaturing conditions. The inability of the antibody to immunoprecipitate protein under denaturing conditions suggests low affinity of the antibody for PLD.

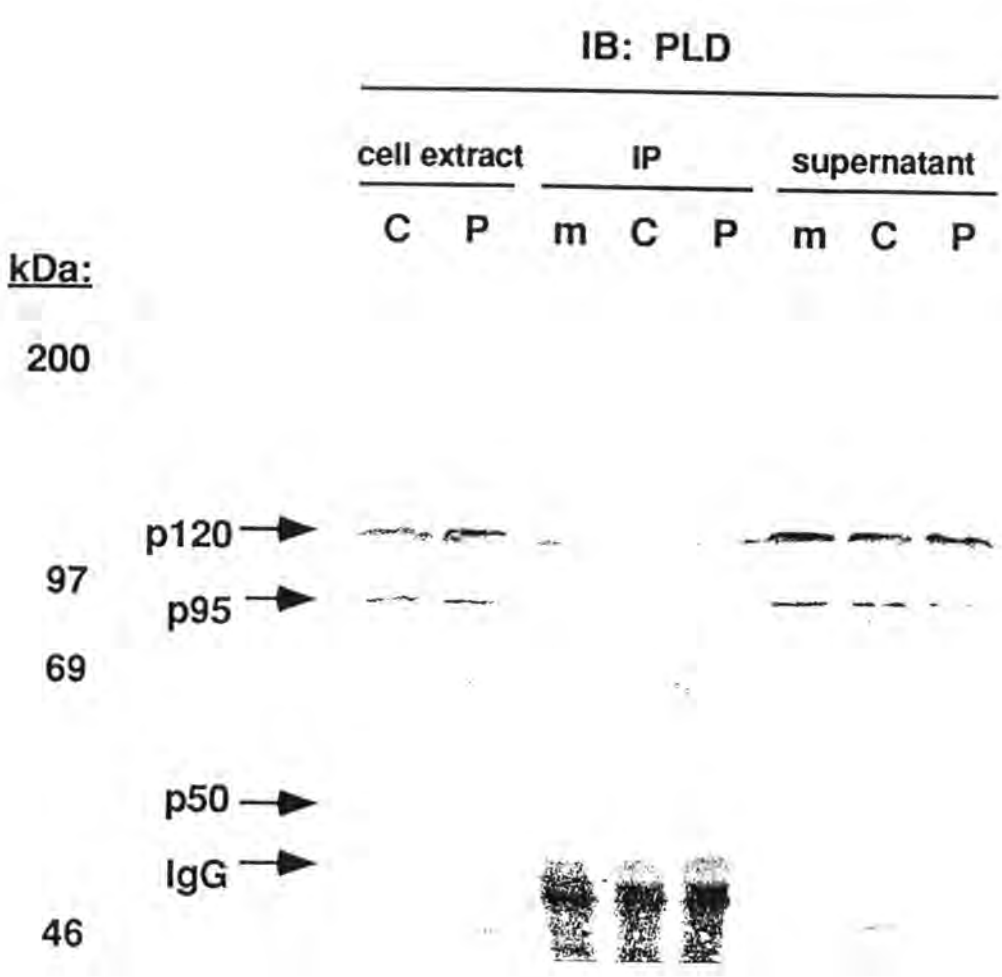

FIGURE 4-4. Immunoblots for PLD in PLD immunoprecipitates.

PLD was immunoprecipitated under non-denaturing conditions from whole-cell extracts prepared from PC-3 cells that had been incubated with ("P") or without ("C") 100 nM PMA for 10 minutes. Lanes labelled ("m") were mock immunoprecipitations of untreated cells performed without PLD antibody. The immunoprecipitates, and the immunoprecipitation supernatants, were subjected to immunoblotting for PLD. Whole-cell extract from untreated cells were also immunoblotted with anti-PLD antibody (far left lane). The position of the major immunoreactive PLD bands, PKCα, and IgG are indicated by arrows. The positions of molecular size markers are indicated on the left.

An alternative approach to analyzing potential interactions between PLD and PKC interactions was tested. PKCα was immunoprecipitated from whole-cell extracts. The immunoprecipitates were immunoblotted with the anti-PLD antibody to determine whether PLD associated with PKCα. In the experiment shown, PMA slightly enhanced levels of the 95-KDa species in PKC immunprecipitates (Figure 4-5). However, the amount of immunoreactive PLD proteins detected in anti-PKCα immunoprecipitates was not consistently increased by PMA treatment. While the IgG signal obscures detection of the 50-KDa band in this particular blot, no evidence for association of this protein with PKCα was obtained in additional experiments.

PKCα immunoprecipitates were also analyzed for PLD activity, using the *in vitro* fluorescent assay. Although PLD activity could be detected in the immunoprecipitates, there was no significant increase in PLD activity in anti-PKCα immunoprecipitates from PMA-treated cells (Figure 4-6). Taken together, these results suggest that PMA does not enhance or promote the direct association of PKCα with PLD isoforms in PC-3 cells.

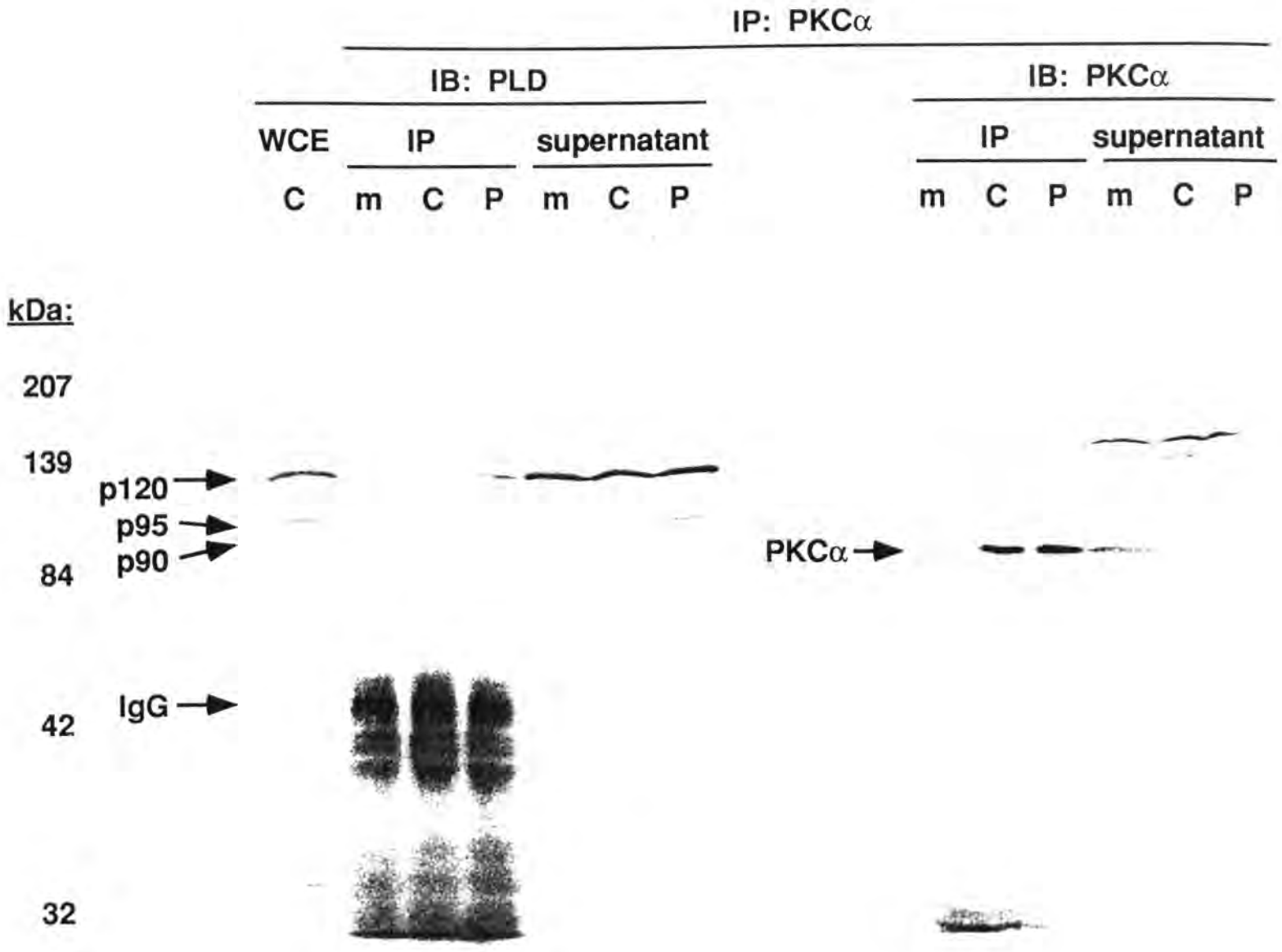

FIGURE 4-5. Immunoblots for PLD in PKCα immunoprecipitates.

PKCα was immunoprecipitated from whole-cell extracts prepared from PC-3 cells that had been incubated with ("P") or without ("C") 100 nM PMA for 10 minutes. Lanes labelled "m" were mock immunoprecipitations performed without PKCα antibody. The immunoprecipitates, and the immunoprecipitation supernatants, were subjected to immunoblotting for PLD and PKCα. Whole-cell extract from untreated cells were also immunoblotted with anti-PLD antibody (far left lane). The position of the major immunoreactive PLD bands, PKCα, and IgG are indicated by arrows. The positions of molecular size markers are indicated on the left. Similar results were obtained in seven different experiments.

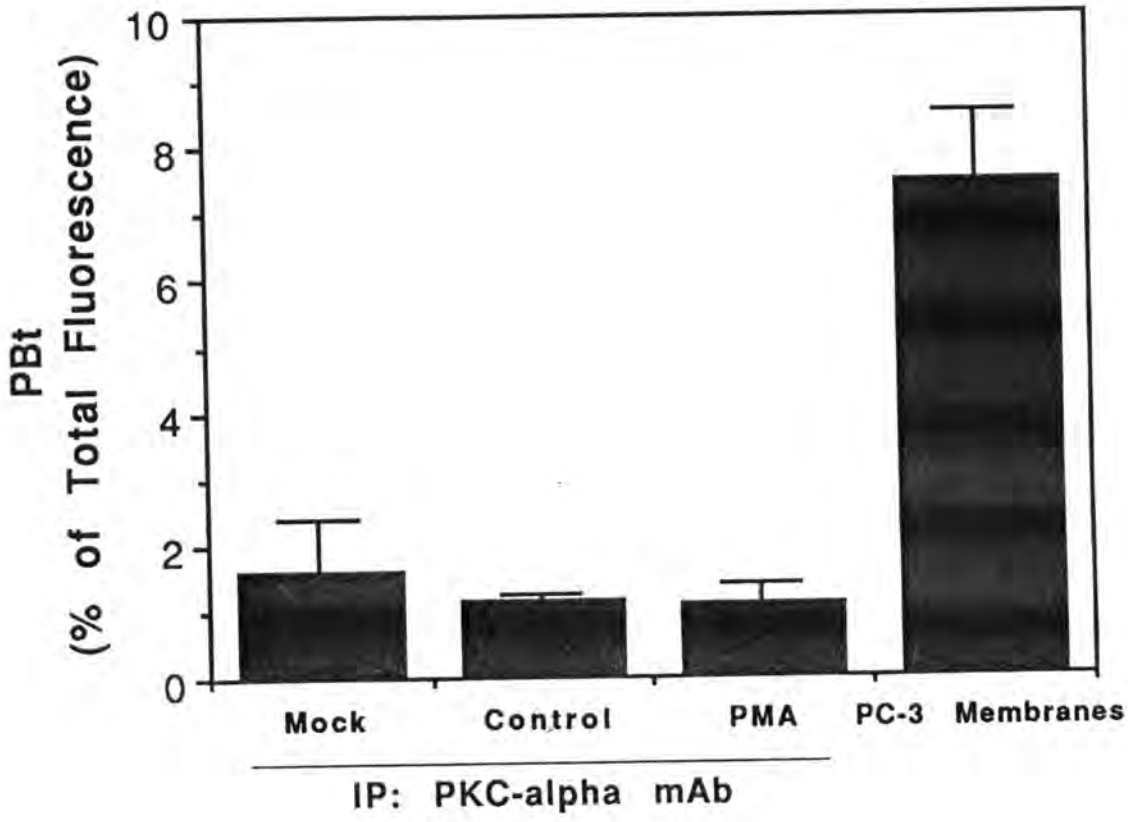

FIGURE 4-6. PLD activity in PKCα immunoprecipitates

PKCα was immunoprecipitated from whole-cell extracts prepared from PC-3 cells that had been incubated with or without 100 nM PMA for 10 minutes. Mock immunoprecipitations were performed without PKCα antibody. The immunoprecipitates were resuspended in lysis buffer and PLD activity was assessed using the *in vitro* fluorescent assay. Data are expressed as the percent of total fluorescence present in phosphatidylbutanol (PBt) band. Similar results were obtained in two separate experiments.

4-4. Discussion

Previous studies have shown that PLD activity is expressed in a number of mammalian cell systems. PLD is generally activated in response to phorbol esters that activate PKC. In this study, we have examined some of the potential mechanisms by which PKCs may activate PLD.

We have attempted to determine whether protein phosphorylation is involved in the activation of PLD. We have characterized the expression of PLD using an anti-PLD antibody in Jurkat, PC-3 and LNCaP cells. Using immunoblotting techniques, we have found that each cell line expresses a 120-KDa PLD that may represent the cloned human PLD1 (Hammond *et al.*, 1995). Confirmation that this protein is indeed hPLD1 will depend on the development of additional anti-PLD antibodies and/or use of RT-PCR to confirm expression of hPLD1 in PC-3 cells. The 95-kDa band may represent a form of PLD similiar in size to the plant enzyme (Wang *et al.*, 1994). In addition, we have identified a 50-kDa band that may be an additional isoform of PLD. When PLD was partially purified from mouse lung membranes, a 50-kDa immunoreactive band eluted in fractions containing PLD activity (Ella *et al.*, submitted). While it

is possible that this band represents a proteolytic product, this seems unlikely in view of the inclusion of multiple protease inhibitors in the tissue extracts.

Our studies using alkaline phosphatase did not provide evidence for a role for dephosphorylation in PMA-induced PLD activity. Although alkaline phosphatase acts as a general protein phosphatase, it is possible that it does not dephosphorylate residues involved in the regulation of PLD activity. Use of other phosphatase preparations or incubation protocols may circumvent the problem of non-specific decreases in PLD activity. A more direct approach to examining PLD phosphorylation would be to use anti-PLD antibodies in immunoprecipitate PLD from [^{32}P]-labeled cells. This approach must await development of immunoprecipitating antibodies. Alternatively, epitope-tagged PLDs could be immunoprecipitated from [^{32}P]-labeled cells transfected with constructs encoding the tagged enzyme. Therefore, further approaches will be required to determine if phosphorylation, or any other covalent protein modifications, are involved in the activation of PLD. The 120-kDa and 50-kDa bands seen in Jurkat and PC-3 cells did not shift in electrophoretic mobility following PMA treatment. Although some

signal transduction proteins shift when phosphorylated, others do not. Therefore, these results further suggest, but do not prove, that protein phosphorylation may not be involved in the regulation of PLD. Until additional mammalian forms of PLD have been sequenced, it will not be possible to establish that all of the bands observed represent PLDs.

This study has also examined the interaction of PLD with PKCα. Our observations provided no direct evidence for association of PKCα with PLD. Several factors may be responsible for these results. First, the anti-PKCα antibody used may not permit co-precipitation of PKCα and PLD under the conditions used. Second, since it has been suggested in the literature that several isoforms of PLD exist (Hammond *et al.,* 1995; Morris *et al.,* 1996), it is possible that the forms of PLD recognized by the antibody in PC-3 cells do not require or interact with PKC for activation. Experiments are in progress in our laboratory to define the form(s) of PLD expressed in Jurkat and PC-3 cells. Third, it has not been established that PLD is selectively regulated by PKCα in this cell line.

While further studies will be required to elucidate the mechanism by which PKC regulates PLD in mammalian cells, our results are consistent with the general hypothesis that PLDs are regulated via G-protein interactions or other protein-protein interactions.

CHAPTER 5

General Discussion and Future Directions

5-1 Conclusions

Several major conclusions can be drawn from the data presented in the previous chapters. First, It has been clearly established in this work that the expression of phorbol ester-sensitive PLD activity varies between different T-lymphocyte cell lines. Activation of PLD is not required for PKC-mediated ERK activation, and elevation of PA is not sufficient to activate ERKs. This was found to be the case in vascular smooth muscle cells (Jones *et al.,* 1994) and fibroblasts (Knoepp *et al.,* 1996), as well as in T-cell lines (Bradshaw *et al.,* 1996a).

Second, it has been determined that activation of neutral SMase can be detected in EL4 and Jurkat T-cells in response to the antileukemic drug, Ara-C, using an *in vitro* fluorescent assay (Bradshaw *et al.,* 1996b). Ara-C does not result in activation of ERKs, and activates JNKs only after a lag of approximately 1 hour. Thus, it is possible that accumulation of ceramide as a result of SMase activation is a direct or indirect signal for JNK activation.

Finally, these studies showed that translocation of PLD from cytosol to membranes is probably not involved in activation of PLD by PMA. As observed for both Jurkat and PC-3 cells, a 120 kDa

protein recognized by an anti-PLD antibody is constitutively localized to the membrane (Bradshaw et al., 1996a; Qi *et al.*, submitted). Our studies did not establish whether protein phosphorylation was required for PLD or SMase activation. A direct interaction between PLD and PKCα was not detected.

This study is the first to examine the role of PLD and SMase in MAPK activation in T-lymphocytes. Overall, our findings from this project suggest that PLD and SMase are independently activated, and thus represent distinct signaling pathways in T-cells. The results of this study contribute to our understanding of T-cell activation and death. Furthermore, the information obtained from this study provides additional knowledge regarding the basic mechanisms involved in the activation of PLD, SMase, ERKs, and JNKs.

5-2. Implications and future directions

An increasing number of studies have been initiated over the past few years to elucidate the involvement of PLD and SMase in cellular signaling processes. Studies of PLD regulation have suggested a role for PLD in intracellular protein trafficking and mitogenic signaling, and have established that members of the ARF

and Rho families of G-proteins can regulate PLDs (Morris *et al.*, 1996). Studies of the sphingomyelin cycle have implicated SMase in inflammatory responses, differentiation, and apoptotic cell death (Hannun, 1994). Even with the large amount of accumulated knowledge concerning PLD and SMase, further studies are still necessary to understand the roles of these enzymes in biological processes.

In T-cells, very little is known about the roles of PLD and SMase in signal transduction. Therefore, this thesis project addressed the hypothesis that PLD and SMase are involved in T-cell signalling. The goals of this project were to: 1) examine the activation state of PLD using the tumor promoter, PMA, as a model compound for the study, 2) examine the activation state of SMase using an antileukemic drug, Ara-C, as a model compound for the study, 3) examine the role(s) of PLD and SMase in activations of ERKs and JNKs, and 4) examine mechanisms underlying the activation of PLD and SMase.

EL4 (a murine thymoma cell line that is phorbol ester-sensitive) and Jurkat (a human cell line derived from an acute T-cell leukemia) were primarily used as model systems for this study.

Jurkat cells are widely used as a model to examine the requirements of normal T-cell activation. These cell lines differ from normal T-cells in that they do not require IL-2 for proliferation. They differ from each other in their requirements for activation of IL-2 gene expression. Jurkat cells do not respond to PMA alone nor PMA plus IL-1, but do produce IL-2 in response to PMA plus phytohemagglutinin or PMA plus calcium ionophore (Baldari *et al.,* 1991) EL4 cells respond to high concentrations of PMA alone, or to a suboptimal concentration of PMA in combination with either IL-1 or calcium ionophore, to produce IL-2. Jurkat cells thus differ from EL4 cells in that they lack IL-1 receptors and require calcium mobilization for IL-2 gene activation (Baldari *et al.,* 1991). It is thought that IL-2-independent growth in these cell lines results from constitutive activation of a transcription factor, such as NFAT. However, the early signal transduction events observed in response to cytokines or PMA/ionomycin appear analogous to those observed in mature T-cells.

Our studies analyzing PLD and SMase activity have led to the finding that these enzymes are indeed regulated through different signaling systems in Jurkat and EL4. Our studies provide substantial

evidence that a great deal of variablity exists for phorbol ester-induced PLD activity in different T-cell lines. This variability may be due to differences in expression of various PLD isoforms (Chapter 2). We have shown that phorbol ester-stimulated PLD activity is present in human Jurkat leukemic T-cells, but is absent in murine EL4 cells. Calcium, ARF, and/or GTPγs, which are known to be required for some forms of PLD, did not stimulate PLD activity in EL4 membranes. Several other T-cell lines tested, such as HT-2, also lacked phorbol ester-induced PLD activity. Likewise, additional studies in our laboratory have shown that basal PLD activity is absent in human and murine peripheral T-cells (Ella *et al.,* 1994). These data are similiar to those obtained by Kinsky and co-workers (1989), who showed that human peripheral blood lymphocytes lacked phorbol ester-stimulated PLD activity. On the other hand, work in our laboratory showed high levels of PLD in mouse thymus (Ella *et al.,* 1994). Since thymocytes comprise the bulk of thymic tissue, these results suggest that the expression of PLD activity is terminated at some point during T-cell differentiation.

Studies of the regulation of PLD have been complicated by the likelihood that multiple isoforms of PLD exist in both cytosol and

membranes of mammalian cells (Siddiqui *et al.,* 1995). Studies have suggested at least two subclasses of PLD enzymes. These include ARF-activated and Rho-activated PLD enzymes, and those enzymes that are sensitive to fatty acids, such as oleate (Morris *et al.,* 1996). Thus far, genes encoding PC-specific PLD activity in plants, PC-hydrolyzing PLD1 in yeast, and PIP_2/ARF-activated hPLD1 in humans have been isolated and cloned (Wang *et al.,* 1994; Morris *et al.,* 1996; Hammond *et al.,* 1997; Ella *et al.,* 1996). Using this information, our laboratory raised an anti-PLD antibody corresponding to residues found in yeast PLD1 and hPLD1. We were able to detect several immunoreactive proteins of molecular weight 120, 95, and 50-kDa in Jurkat and HL-60 cells (Chapter 2) using the anti-PLD antibody. These proteins could also be seen in the PC-3 and LNCaP prostate cancer cell lines, which express high levels of phorbol ester-induced PLD activity (Chapter 4). Our results from these studies lead to the following conclusions: 1) some cells lack PMA-activated PLD activity and 2) the lack of expression of PLD isoforms in T-cells may be responsible for absence of PLD activity. A better understanding of factors affecting the expression and regulation of PLDs is necessary for future advances in this area.

Some studies have shown PKC-dependent, but phosphorylation-independent, activation of PLDs. We were unable to show any direct evidence for the association of PKCα with PLDs (Chapter 4). Until additional forms of mammalian PLDs are isolated and sequenced, and additional antibodies developed, it will be difficult to establish the mechanisms responsible for PKC-mediated regulation of PLD activity.

Our *in vitro* studies of SMase activity have established that this enzyme is regulated independently of PLD activation in T-cells. This project has revealed that a magnesium-dependent SMase is activated in EL4 and Jurkat T-cells in response to treatment with Ara-C, an anti-leukemic drug. The activity was found solely in membrane preparations and was maximal at neutral pH. SMase activity was not affected by treatment of cells with the PKC activator phorbol ester, or by treatment with calcium ionophore. These results confirm our hypothesis that PLD and SMase are independently activated. The mechanism of SMase activation has not been established. Progress in this area is limited by a lack of molecular information concerning the structure of neutral SMase.

The roles of PLD and SMase activation in the MAPK

phosphorylation cascade have not been clearly defined in T-cells. The results discussed in Chapters 2 and 3 showed a lack of correlation between the activation of PLD or SMase and the activation of ERKs. It is possible that the PA generated by PLD contributes to, but is not required for, Raf activation. Our study shows that ceramide does not affect the ERK phosphorylation cascade in Jurkat and EL4 cells. These results are consistent with the general hypothesis that ceramide generation is anti-proliferative. This hypothesis was further confirmed by the ability of C2-ceramide to partially induce DNA fragmentation, a hallmark of apoptosis, in EL4 cells (data not shown). We were however, unable to detect significant nuclear morphological changes associated with apoptosis in either Jurkat or EL4 in response to Ara-C or ceramides using microscopy techniques.

In T-cells, JNKs are synergistically activated by PKC- and calcium-mediated pathways (Su *et al.*, 1994). We were able to detect a similiar synergism in EL4 cells, which lack PLD activity, upon treatment with PMA and calcium. This suggested that PLD is not required for the activation of JNK. Likewise, the fact that calcium did not induce a synergistic activation of PLD also suggests

that JNK does not play a role in the activation of PLD.

The involvement of SMase in JNK activation was, however, quite different from that of PLD. Even though JNK was not activated at early time points when SMase activation was initiated, we were able to detect JNK activity after prolonged treatment with Ara-C. Since SMase activity remains elevated for prolonged time in EL4 and Jurkat, these results are consistent with recent data suggesting that the accumulation of ceramide may induce cellular responses in non-lymphocytic cells. However, since addition of high concentrations of cell-permeable ceramides did not activate JNKs, the role of SMase activation in Ara-C-induced JNK activation remain unclear. Prolonged activation of JNKs may result in apoptosis, as suggested by work from other laboratories (Parks *et al.,* 1996; Testi, 1996). However, it should be noted that the roles of ERKs and JNKs have not been completely elucidated in T-cells, where JNK activation is associated with proliferation and ERK activation is not required for proliferation.

It is possible that PLD may be involved in proliferative responses in T-cells, while SMase is involved in anti-proliferative responses. Such observations could be beneficial not only in

developing new therapeutic approaches, but in developing a better understanding of apoptosis and growth suppression in T-cells in correlation with pathogenesis. Further studies will be needed to establish the linkage between activations of SMase and JNK in different cell types in order to elucidate the role(s) of SMase in cell death. Determination of the various mammalian isoforms of PLD, inhibitors of the enzymes, and interrelationship of the enzymes with various kinases will be necessary to provide targets for future drug development and clinical advancements. While similar studies of neutral SMase are only in their early stages, this enzyme is likely to emerge as an important clinical target in the future.

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